PLANT GALLS: A MODEL SYSTEM TO STUDY PLANT DEVELOPMENT

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ABSTRACT

Cynipid gall formation is achieved by the intimate insect-plant interaction where by cynipid wasps redirect host plant development to form novel structures to protect and nourish the developing larva. To investigate the molecular mechanisms involved in this interaction, and extend our understanding of plant development, four approaches were taken.

1) A PCR based approach to search for genes to known signalling molecules: chitiooligosaccharides, or Nod factors, that control nodulation in the Rhizobia-legume interaction. PCR analysis was used to investigate the presence of the *nodC* gene in the cynipid gall wasp genome, however, no *nodC*-like sequences were found.

2) SDS-PAGE analysis was carried out to compare inner-gall and non-gall protein signatures, demonstrating the variation between gall and non-gall tissue, and also that the protein signatures of inner-gall tissues vary between gall species. N-terminal sequencing and western blot analysis lead to the identification of a number of inner-gall proteins such as protein disulphide isomerase (PDI), formate dehydrogenase (FDH) and putative biotin carboxyl carrier protein (BCCP), involved in the synthesis of lipids in seeds. Analysis of the temporal and spatial expression of the putative BCCP revealed expression to be concentrated in the inner-gall cells throughout development, in all the gall species tested.

3) Cytological analysis of the inner-gall tissue was carried out throughout development of several gall species to investigate differences in their patterns of development and cytological characteristics of the inner-gall tissue, with many inner-gall cells being polytene.

4) A gall formation bioassay, to enable the activity of possible signals involved in gall formation to be tested, was developed. Rose callus tissue was used as a test tissue and the cynipid larval extract was exposed to this as a source of the active molecules. The induction of proteins in the callus after exposure to the larval extract was used as a molecular marker for activity.

The polytene characteristic and the possible expression of seed proteins, suggest that seed developmental pathways may be used during gall formation.

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List of Abbreviations

DAPI	4', 6-diamidino-2-phenylindole dihydrochloride
cDNA	complementary DNA
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
g	grams
h	hours
IPTG	isoproyl-_-D-thiogalactopyraoside
kb	kilobases
kDa	kilodaltans
kg	kilograms
L	litre
M	molar
mg	milligram
min	minutes
µl	microlitre
ml	millilitre
µm	micromolar
mM	millimolar
mm	millimeter
mRNA	messenger ribonucleic acid
ng	nanogram
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	seconds
UV	ultra violet light
V	volts
w/v	weight: volume ratio
X-gal	5-bromo-4-chloro-3-indolyl-_-D-galactopyranoside

1: Introduction

1.1 Gall induction

Long established biotic interactions with plants demonstrate the control that insect, bacterial and fungal signals can have over normal plant development. The evolutionary arms race where plants evolve defences against herbivorous insects, bacteria and fungi and how these evolve strategies to overcome those defences has long been a field of intensive research (Feeny, 1968; Kúc, 1997). Within this spectrum of interactions, a taxonomically diverse group of herbivorous insects evolved the ability to alter plant development and physiology to result in the formation of galls. Among the gall-inducing insects, cynipid gall wasps (Hymenoptera: Cynipidae) arguably elicit the development of the most complex galls, which have been referred to as novel plant organs, to protect and feed the developing larva (Crespi 1997). Figure 1.1 shows four cynipid galls, demonstrating the diversity of external morphology between galls. The earliest records of cynipid galls go back to Hippocrates 406-347 B.C. and Theophrastus 371-286B.C., although these authors were unaware that they were induced by insects (Felt, 1936, 1940). The first description of the plant gall-former interaction and of a mechanism by which the plant is induced to form a gall was suggested by Malpighi. He believed that the formation of galls was controlled by a fluid deposited with the egg during oviposition and suggested that the fluid reacted with the host's sap and thus controlled the formation of the gall (Malpighi, 1686). Similarly, Darwin (1872) stated that the formation of such varied and species-specific structures was caused by gall wasps each having different types of poison, all inducing gall formation but with species-specific morphologies. In contrast to a fluid applied by the ovipositing female, Alders (1880) and Beyerinck (1882) both suggested that the larvae were the source of the morphogenetic signal for gall formation. Based on

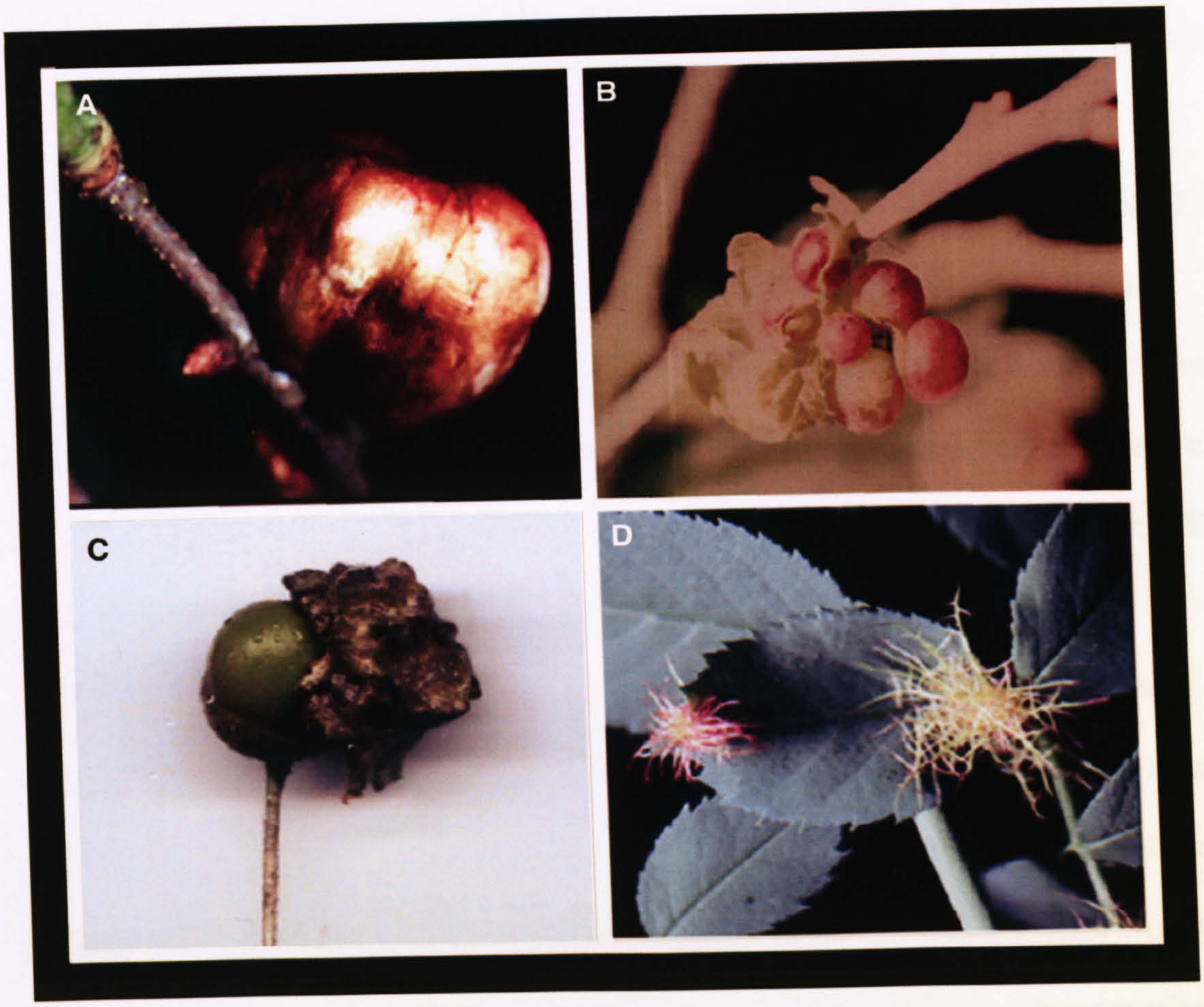


Figure 1.1 External morphologies of four cynipid galls formed on oak and rose.

A) *Biorhiza pallida* bud gall formed on English oak. B) *Neuroterus quercusbaccarum* leaf gall formed on English oak. C) *Andricus quercuscalicis* acorn gall formed on English oak. D) *Diplolepis rosae* leaf gall formed on rose.

observations that gall formation did not begin until the larvae hatched from the egg, Alders thought that it was the mechanical stimulus from the feeding larva that was responsible for the changed developmental pattern in the host. Beyerinck, however, believed that gall formation begins during the egg stage and that the larvae inside the egg produced a signal controlling gall formation. He demonstrated that the development of galls stopped if the eggs were destroyed before the larvae hatched or if larvae were killed.

To date it is generally believed that as yet unidentified morphogenetic signals from the cynipid larvae are of a chemical nature although it has been suggested that chemical substances exuded from the egg might also have a role (Bronner, 1977; Rohfritsch and Shorthouse, 1982). While the insight gained by these early studies is very impressive, little is understood as to how cynipids are able to reprogramme plant development. The understanding of other plant developmental processes, however, has progressed immensely with the development of modern methods in biochemistry and molecular biology. It therefore seems appropriate to reevaluate and investigate the gall formation process and its underlying molecular mechanism using modern techniques. Identification of signalling mechanisms used by the insect to hijack the plant's development will provide a greater insight into signalling during plant development and the consequences when this is interrupted. Cynipid galls were chosen for this investigation due to their specific differentiation into distinct tissue layers, common throughout all cynipids. This enables gall specific characteristics to be identified, a challenge in non-cynipid galls where some are composed simply of parenchymatous cells.

In this chapter, I will begin by re-examining evidence for proposed morphogenetic signals

involved in gall induction, much of which has been carried out on non-cynipid galls, and review it in the light of known plant signalling pathways. I will then introduce cynipid galls and discuss possible signals involved in their induction and formation. Nematode and rhizobial interactions with plants will be discussed to highlight our understanding of other biotic-plant interactions and how this has influenced the direction taken in our research in gall formation. A version of this chapter is to be published as a book chapter entitled "*Mechanisms and effects during cynipid gall formation: a review*" (Harper, L.J., Schönrogge, K., Lichtenstein, C.P.) In: The biology and biogeography of palaearctic gall wasps (Hymenoptera: Cynipidae). (Ed. Stone, G.N.) (in press).

1.1.1 Morphology and evolution

Ancestral cynipids are believed to have induced swellings on herbaceous plants such as *Papaver* (Liljeblad and Ronquist, 1998). From there cynipids have evolved to induce extremely complex structures involving specialised tissues, supplied with their own vascular system and possessing surface structures such as hairs, spines or glands (Liljeblad and Ronquist 1998). The morphologies of the induced galls are specific to the cynipid species, and where the species has a heterogonic lifestyle, alternate sexual and parthenogenic generations, they are specific to each generation. In fact, it is often easier to identify the inducing cynipid species by their galls rather than by adult morphology. The selection pressures, which have lead to the variation in gall morphology are uncertain, and a number of hypotheses have been proposed about the adaptive value of gall formation (Price *et al.*, 1986). A feature common to most cynipid galls is the occurrence of so-called nutritive tissue, a highly nutritious tissue, which provides the actual food for the gall wasp larva. While the selective benefit of being able to induce the production of high quality

food to the gall wasp is intuitively obvious (the nutritive hypothesis), and might have contributed to the evolution of a gall-forming life history trait, it does not address the evolution of such varied morphological characteristics as the structure of the gall itself.

The enemy hypothesis states that gall formation was selected for, to create "enemy free space" for the gall wasp larva, providing protection from natural enemies such as parasitoid wasps; These lay their eggs in the larvae of the gall former, where they develop and consume the host's tissue, killing the gall former (Price *et al.*, 1986). Gall size, surface structures and other gall traits are believed to act as defences against natural enemies and predators. The elaborate morphological structures largely consist of cortical tissues and epidermal tissues that can develop into hairs, spines or glands. Internally, the cortical layers of cynipid galls contain frequently high concentrations of tannins, which is in contrast to the inner nutritive layer (Berland and Bernard, 1951; Baggato and Shorthouse, 1994). The high tannin content is thought to protect the gall from free feeding insect herbivores, in which the tannins might reduce growth and survival, and also act as a fungicide (Feeny, 1968; Taper and Case, 1986,1987). Today, however, most cynipids are hosts to parasitoid wasps and are prey to a variety of predators. Thus, the interpretation of gall structures as defences today is based on indirect evidence. Studies on the parasitoid communities associated with cynipid galls and on the evolution of gall structures suggest that these can only be overcome by particularly adapted parasitoid and predator species (Askew, 1965; Price *et al.* 1987; Schönrogge *et al.*, 1998, 1999; Stone *et al.* 1995; Stone and Cook, 1998). For example the *Torymid* parasitoids have long ovipositors, able to pierce through the large cortex of the gall to reach the gall former in the centre.

1.2 Non-cynipid gall formers

A large number of studies, and particularly more recent studies, have investigated the gall formation process in non-cynipid systems, especially aphids (Homoptera: Aphididae), gall midges (Diptera: Cecidomyiidae) and sawflies (Hymenoptera: Tenthredinidae) (Beyerinck, 1882; Boysen-Jenson, 1948; Anders, 1957, 1958; McCalla, 1962; Schäller, 1963, 1968; Hori and Miles, 1977; van Staden and Davey, 1978, 1977; Tandon and Ayra, 1980; Higton; 1994; Leitch, 1994). Although the galls induced by these gallers are morphologically simpler than cynipid galls, one might argue that there are only a limited number of ways to manipulate plant development, and that at least some aspects of the gall induction processes in these different groups should be similar. Morphologies of galls induced by sawflies, midges, aphids and mites can be seen in Figure 1.2. The sawfly gall, *Potania proxima*, shown in A, shows a red bean shape leaf gall formed on willow. The gall shows very simple morphology formed from proliferation of cells from the upper and lower leaf. The midge gall, *Macrodiplosis dryobia*, in B is formed on oak by the folding of the leaf edge, caused by increase proliferation on one side. The aphid gall, *Pemphigus bursarius*, in C shows a pouch gall in the petioles of black poplar and the mite gall, *Eriophyes tristriatus*, shown in D is a blister –like swelling on the leaf. All these non-cynipid galls show simple external and internal morphology with no distinct tissue layers emphasising the complexity of cynipid galls. To investigate gall formation in these non-cynipid systems the main approach taken has been the use of a bioassay (Anders, 1957, 1958; McCalla *et al.,* 1962; Schäller 1963, 1968; Higton, 1994). This tests the activity of extracts such as salivary glands extracts or larval secretions by exposing the extract to living tissue and observing increased growth or callus formation as a marker for activity. These assays are very simple and although they do assess activity of an extract to some

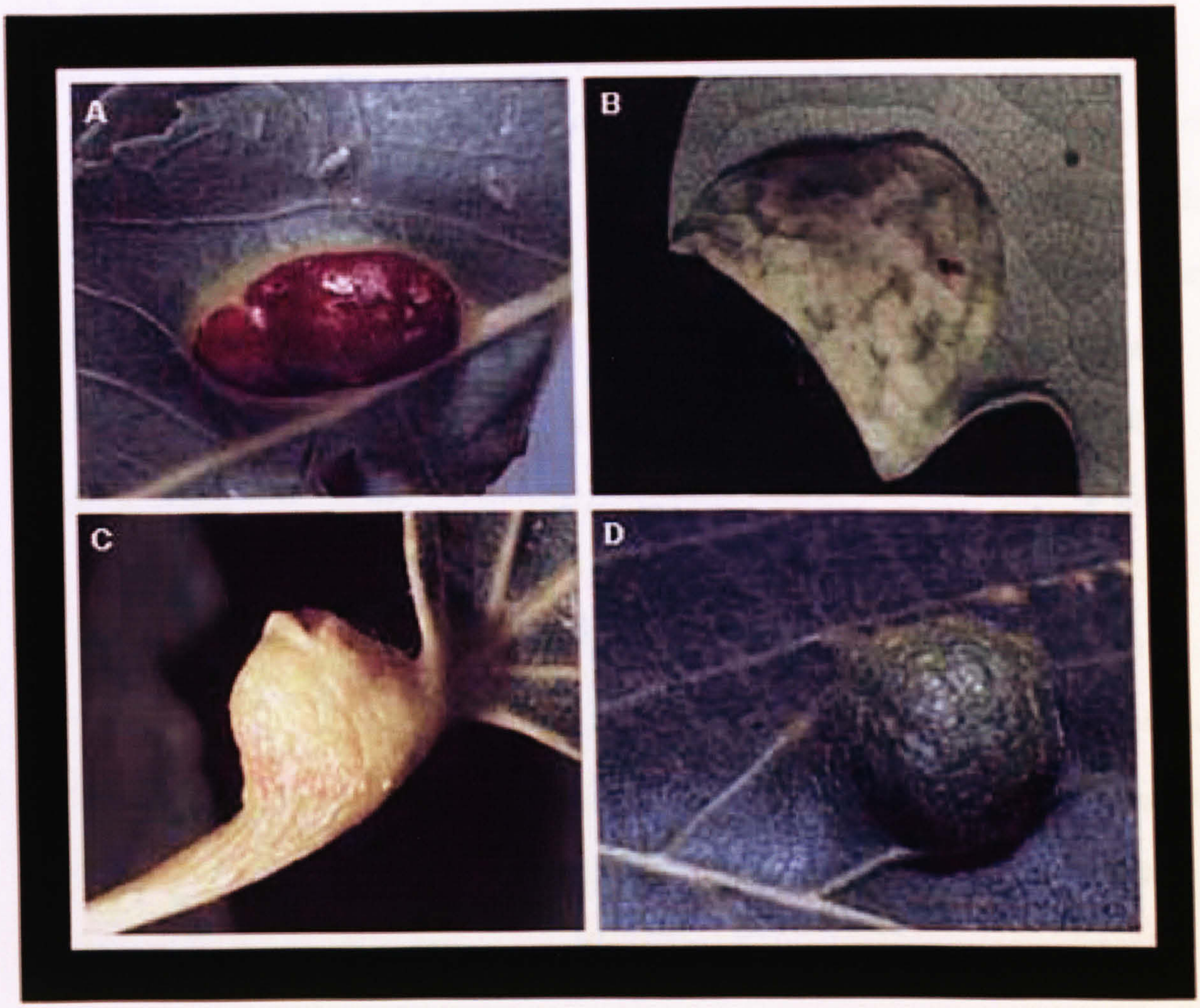


Figure 1.2 External morphologies of four non-cynipid galls. A) *Pontania proxima.* B) *Macrodiplosis dryobia.* C) *Pemphigus bursarius.* D) *Eriophyes tristriatus.*

degree, gall formation related activity is not specifically assessed. An assay specific to gall formation is required to identify gall formation signals; this will be discussed further in chapter 6.

To investigate gall formation in these non-cynipid systems the main approach taken has been the use of a bioassay (Anders, 1957, 1958; McCalla *et al.,* 1962; Schäller 1963, 1968; Higton, 1994). This tests the activity of extracts such as salivary glands extracts or larval secretions by exposing the extract to living tissue and observing increased growth or callus formation as a marker for activity. These assays are very simple and although they do assess activity of an extract to some degree, gall formation related activity is not specifically assessed. An assay specific to gall formation is required to identify gall formation signals; this will be discussed further in chapter 6.

As the investigations into gall formation involve the study of plant responses, plant defence response needs to be considered. A plant's reaction to external foreign substances can have several forms. Defence related compounds produced in response to pathogens or wounding of the plant include proteases, chitinases, phytoalexins, phenoloxidases and oligosaccharides (Kúc, 1997). They are non-specific and aid in disease resistance by digestion of pathogens (Kúc, 1997). Callus formation can also be a response to wounding; however, in a wound response necrosis of the cells surrounding the wounded area usually follows protecting the plant from further damage. A wound response is not always caused during the process of oviposition, although one is induced in response to the egg. To achieve gall induction, the egg escapes degradation by the wound response and perhaps even uses the signals induced to its own advantage.

Many of the studies on gall formation have been carried out on non-cynipids and therefore, first I will discuss the research carried out on non-cynipids. By highlighting the work achieved in this area, it provides a more detailed understanding of how insects "talk" to plants and may give an indication of the possible signalling pathways used during the more complex cynipid gall formation.

1.2.2 Midge galls

The stimulus provided by the larva of gall midges, and therefore possibly all gall forming insects, was shown to be of a chemical nature and not a mechanical stimulus by Boysen-Jenson (1948). He demonstrated that the beech gall induced by Cecidomyiidae *Mikiola fagi* still formed even when a layer of lanolin paste was placed between a single larva and the host tissue, preventing any physical contact but allowing diffusion of chemical compounds. The presence of the larva stimulated an increase in cell proliferation in the host tissue, demonstrating that a diffusable compound was causing the proliferation. The active compound was also demonstrated to cause cell elongation: when paste which had been between the larva and plant was placed on a leaf, unilateral growth was observed (Boysen-Jenson 1948).

1.2.3 Sawfly galls

In the sawfly *Pontania proxima,* it has been demonstrated that gall-inducing substances are contained in the ovipositional fluid secreted by accessory glands in the female's oviduct during egg laying. The secretion coats the egg and is used to help attachment when deposited. When a female inserts her ovipositor into the host plant but lays no eggs,

an incomplete but still recognisable gall is formed (Bayerinck 1882). For complete gall formation to occur, the larva must be present (McCalla *et al.,* 1962). McCalla *et al.* (1962) used extracts of adult *Pontania* accessory glands in a bioassay experiment to show gall-inducing activity and to try to identify the active compound, shown in Figure 1.3. The bioassay involved injecting extracts into growing galls, from which the larva had been removed, and measuring the proportional growth of the gall over a period of time after a series of injections. Compared to negative controls the greatest relative gall growth was observed when the extract was injected. Further treatments of the accessory gland extract demonstrated that the active compound is of low molecular weight. Paper chromatography separation revealed that there were six main compounds present in the accessory gland extracts and of these glutamic acid, uric acid and two adenine derivatives were present in significant concentrations and together induced growth in the bioassay (McCalla *et al.,* 1962). Higton (1994) pointed out that since McCalla *et al.* worked with maturing galls the compounds identified by them might be relevant to gall growth but possibly not gall induction.

Higton (1994) also analysed the accessory gland extract in *Pontania* by using a callus growth bioassay based on the amount, longevity and pigmentation of induced callus, shown in Figure 1.3. This involved injecting the accessory gland extract into an upper leaf vein, simulating a *P.proxima* oviposition, and comparing the induction of cell proliferation forming callus, to a negative control of distilled water. This enabled a standard wound response, in which the callus soon undergoes necrosis, and the response to the extract to be separated. The colecteral fluid induces a long-lived callus with some red pigmentation. Red pigmentation of callus was used as a marker in the bioassay, as the actual gall has a

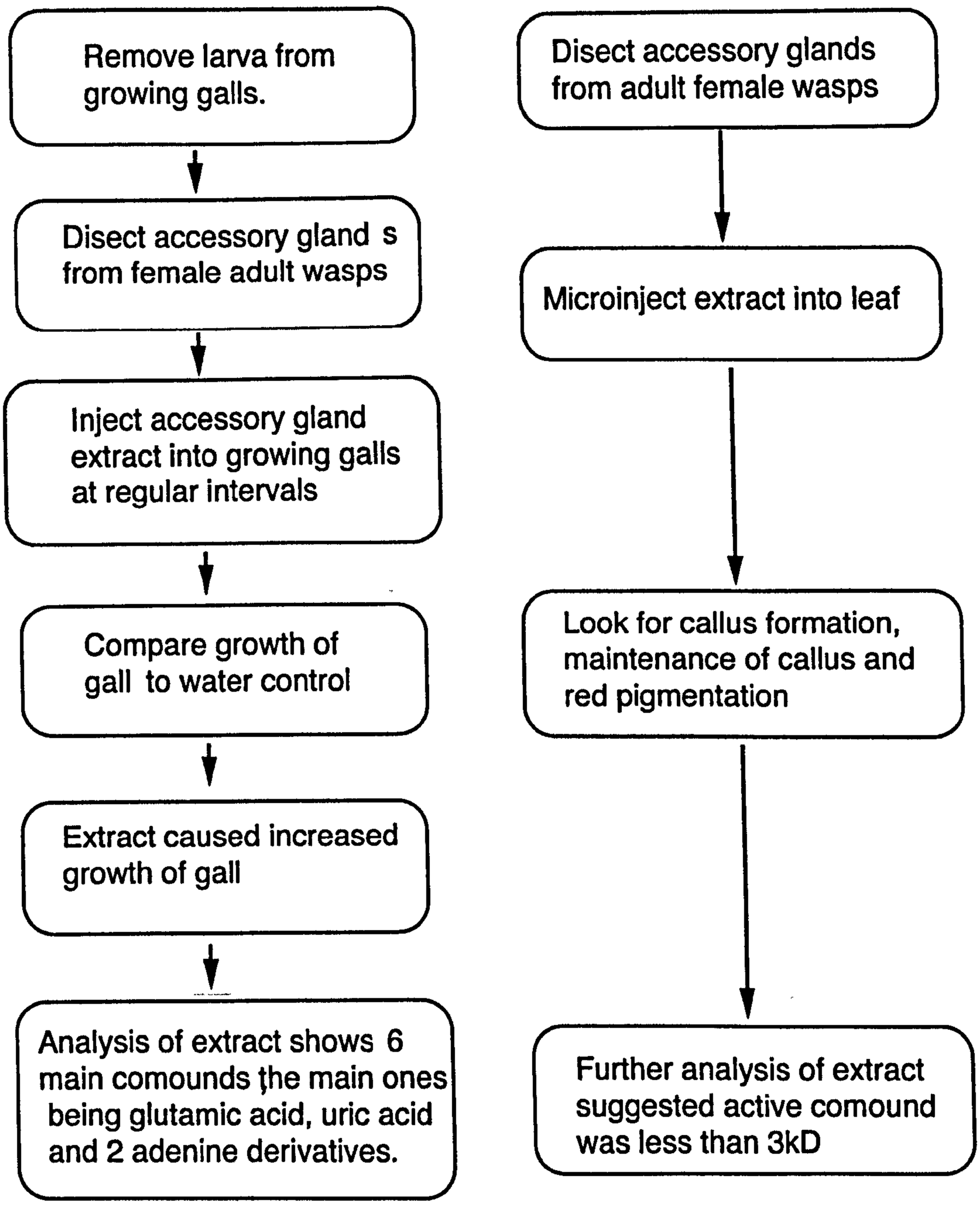


Figure 1.3 The protocols of bioassays used to assay accessory gland extract.

characteristic red pigmentation on the upper side of the leaf. Higton (1994) used ultrafiltration and found that the active fraction contained molecules less than 3kD and had a UV absorption peak at 247nm.

The two different bioassays carried out by McCalla *et al.* (1962) and Higton (1994) test similar extracts and demonstrate that growth-promoting compounds are contained within the extract. The increase in growth of an established gall and the formation and maintenance of callus do demonstrate an increase in cell proliferation. Whether increase in proliferation is sufficient to suggest gall initiation might be arguable, although the red pigment is more significant.

1.2.4 Chalcid galls

To achieve gall formation, plant growth hormones are likely to be involved; how the insects manipulate these and in what combination these are used is unknown. Chalcid galls, induced on the leaves of the broad-leaved coral tree (*Erythrina latissima),* were studied for the concentrations of endogenous cytokinins (van Staden and Davey, 1978). Leaf laminae, galls and larva were used from all stages of development and an analysis carried out using a soybean callus bioassay and paper chromatography (van Staden and Davey, 1978). Cytokinin concentration was found to be lower in the intact gall than in the leaf and accumulated in both throughout development (van Staden and Davey, 1978). Most of the gall cytokinin was located within the larva itself while the actual gall tissue contained very little. The larva contained more of the active form of cytokinin, zeatin and zeatin riboside, whereas the leaf laminae contained mainly a storage cytokinin, zeatin glucoside. In mature galls, the larvae did not contain cytokinin, leading van Staden and

Davey (1978) to suggest that the larva is not producing the cytokinins itself, but obtaining them from the host and transforming them into the active form to aid gall development. If cytokinins are responsible for the cell proliferation then specific distinctive tissue layers within cynipid galls would probably require an additional signal.

1.2.5 Aphid galls

Aphid galls are simple, appearing as a swelling or rolled leaf. Analysis of the salivary gland secretion of aphid gall former *Viteus vitifolii* revealed that the main constitiuents were amino acids: glutamine, asparagine, serine, alanine, and aspartic acid (Miles, 1968; Schäller, 1968). Schäller (1963, 1968) also noted that the amino acid composition and concentration varied between species. Amino acids corresponding to those found in aphid salivary glands have been shown to actively induce a response when used in bioassays (Schäller, 1963, 1968; Anders, 1957, 1958). Vine seedlings grown in a medium containing these amino acids developed simple nodules on the roots (Anders, 1957,1958; Schäller, 1968). Schäller (1968) described induction of a more natural gall-like structure when an amino acid and auxin mix was used. This led Schäller (1968) to the conclusion that auxins have an important role in gall induction. However, since auxin concentrations in the aphids were found to be significantly lower than in the gall tissue, the aphids are unlikely to be the source of the auxins (Hori, 1992).

1.2.6 Hemipteran galls

Gall-inducing hemipteran salivary glands of *Lygus disponsi*, *Poecilometis punctiventris* (Pentatomidae) and *Dindymus versicolor* (Pyrrhocoridae) were analysed by Hori and Miles (1977) and found not to contain any IAA, but when tested on *Avena* coleoptiles with

IAA added to the media, a significant growth of the coleoptile was observed, compared to when just IAA was used. This indicates the presence of IAA synergists, which accelerate the function of this phytohormone (Hori and Miles, 1977). Alternatively auxin protectors, which prevent the destruction of auxin, could cause local increase in IAA concentration.

1.2.7 Mite galls

Gall tissue induced by *Eriophyes* (Acria: *Eriophyidae*) was analysed by Tandon and Ayra (1980) and three auxin protectors were detected, preventing the oxidation of auxin by peroxidase. An increase in the enzyme polyphenol oxidase (PPO), that controls the production of auxin protectors, was observed up to the 30th day after induction (Tandon and Ayra, 1980). This further suggests that the production of auxin protectors within the gall causes an increase in IAA concentration and thus promotes cell proliferation.

From these studies, the control of non-cynipid gall formation remains unclear, although in some cases involves signals secreted by the female adult in addition to chemical signals from the larva. The signals manipulate plant growth hormones to achieve increased plant growth, however, the specific differentiation of each gall probably involves additional specific signals from the larva to achieve complete gall formation, which are still to be identified.

1.3 The morphology and physiology of cynipid galls

Cynipid gall formation is not induced by ovipositional fluid alone as will be discussed in section 1.4.1, although, it is likely to involve the manipulation of plant growth factors. The structure of the cynipid galls is more complex than those discussed previously, made up of several different tissue layers, and probably involves a number or cascade of specific

signals. The formation of cynipid galls will now be discussed. The development and differentiation of tissues during cynipid gall formation, and the physiological conditions within those tissues have been analysed. The understanding of the cytological changes throughout gall formation may indicate the type of signals used by the larva and enable comparison of gall induction to similar processes and conditions that are known from normal plant development.

1.3.1 Inner-gall Morphology

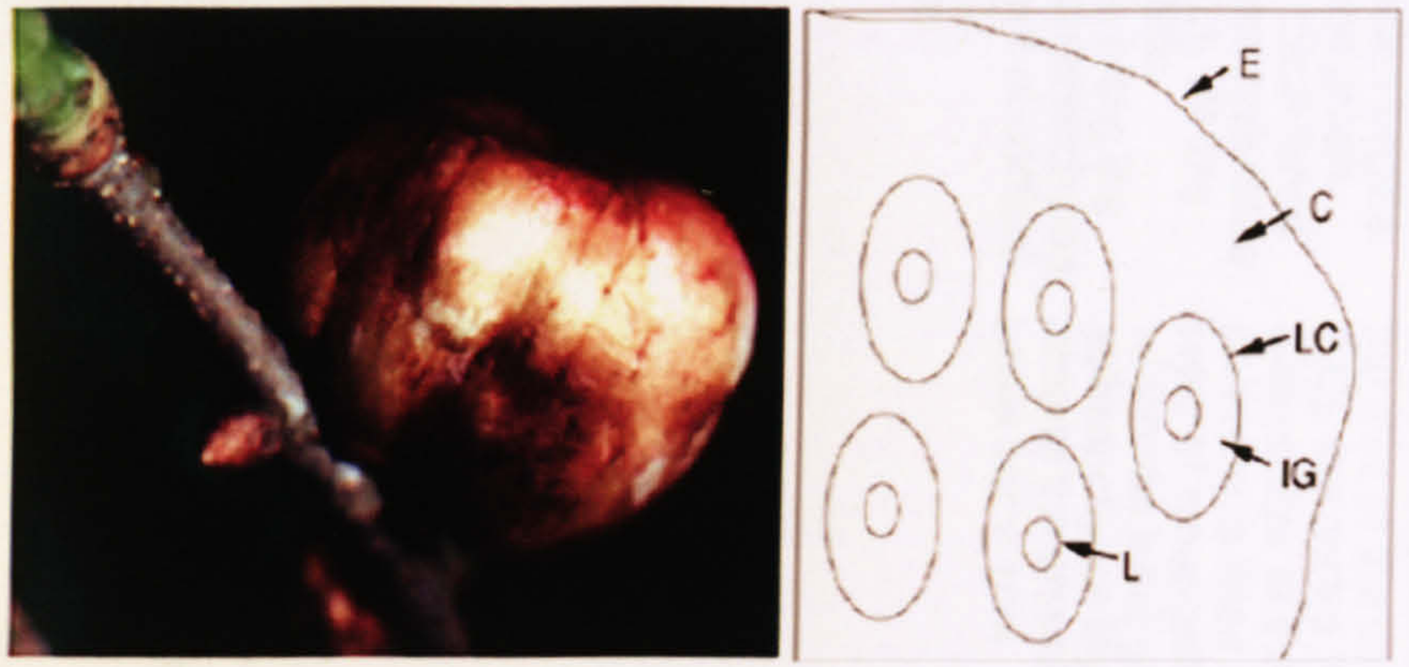
The spatial tissue composition of a cynipid gall consists of an outer epidermis, surrounding a cortical parenchyma, forming the outer-gall. The boundary between the outer and the inner-gall is indicated by a sclerenchyma that encapsulates the nutritive parenchyma and the nutritive tissue, surrounding the larva. In contrast to the outer morphologies, the inner-gall organisation has a general pattern throughout most cynipid galls (see Figure 1.4 for external and internal organisation of four cynipid galls).

The development of a cynipid gall can be divided into three interconnecting phases: 1) initiation, 2) growth and 3) maturation (Rohfritsch and Shorthouse, 1982). Here we revisit the description of phases 1, 2, and 3 as given by Rey (1992), using the multichambered oak apple galls induced by *Biorhiza pallida* on English oak (*Quercus robur)* in spring and *Aylax glechomae,* a round (6-10mm), multichabered leaf or stem gall, induced on ground rose (*Nepeta hederacea),* to demonstrate the responses. *Diplolepis nodulosa* formed on *Rosa blanda* will alsso be discussed, although its development is similar to that of *B.pallida* (Brooks and Shorthouse, 1997,1998). Figure 1.5 shows galls of *B.pallida* at the different stages of development.

Figure 1.4 External and internal morphology of four cynipid galls.

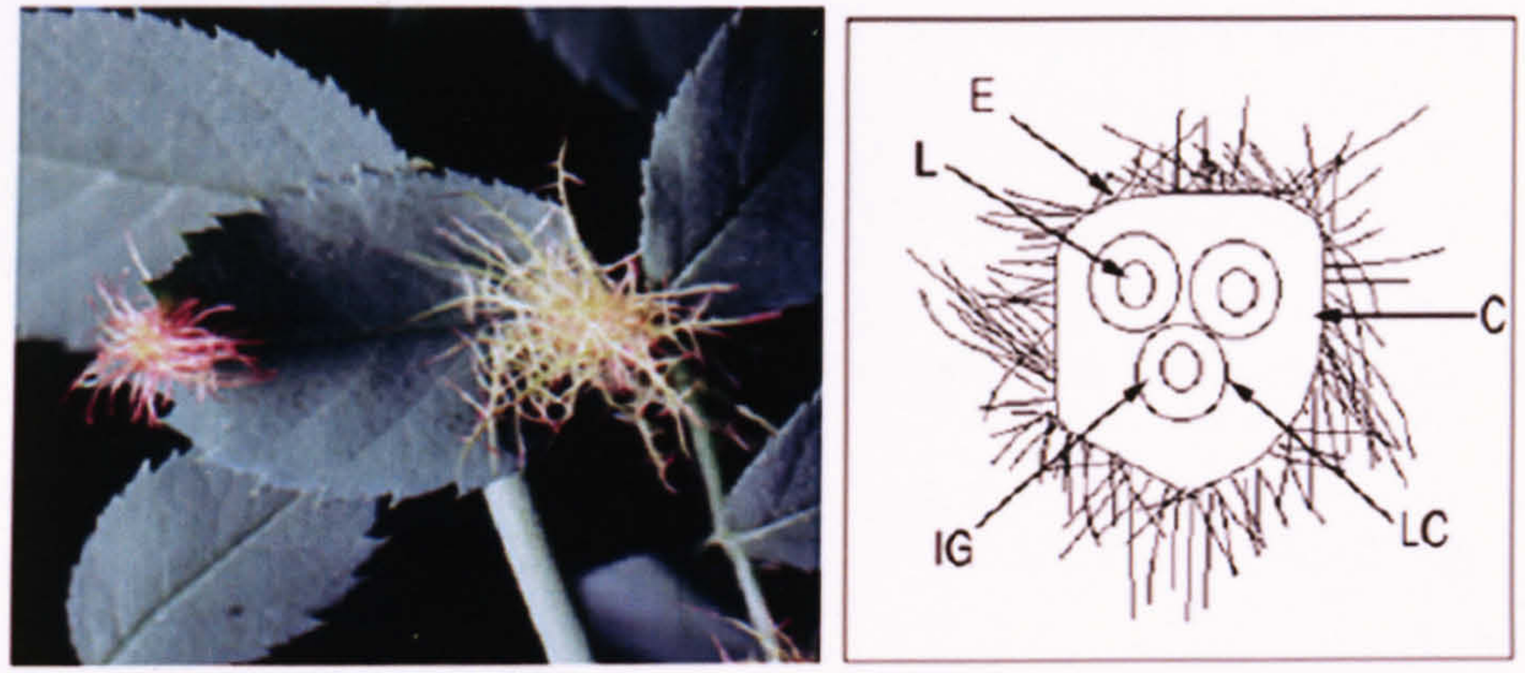
E = Epidermis, C = Cortical parenchyma, LC = Larval chamber
IG = inner-gall tissue, L = Larva

A. *Biorhiza pallida*



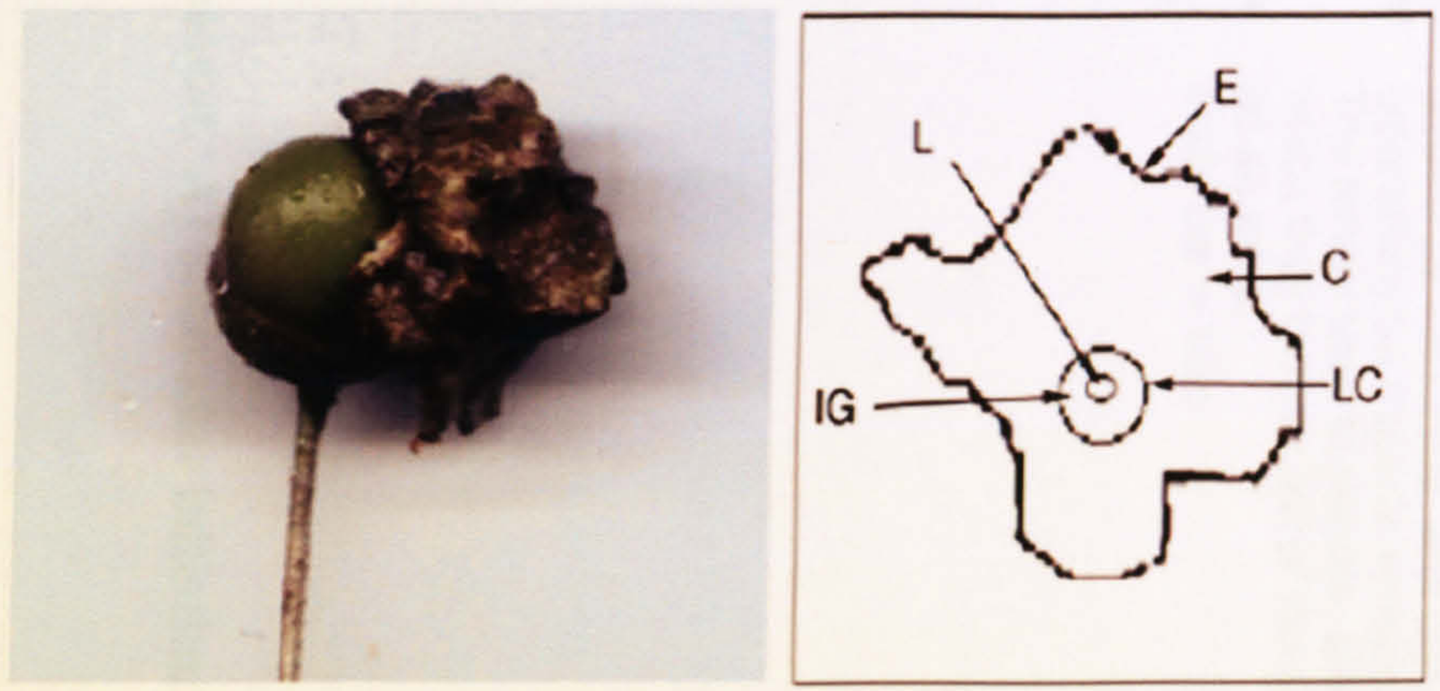
Muti-chambered bud gall
on *Quercus robur*

B. *Diplolepis rosae*



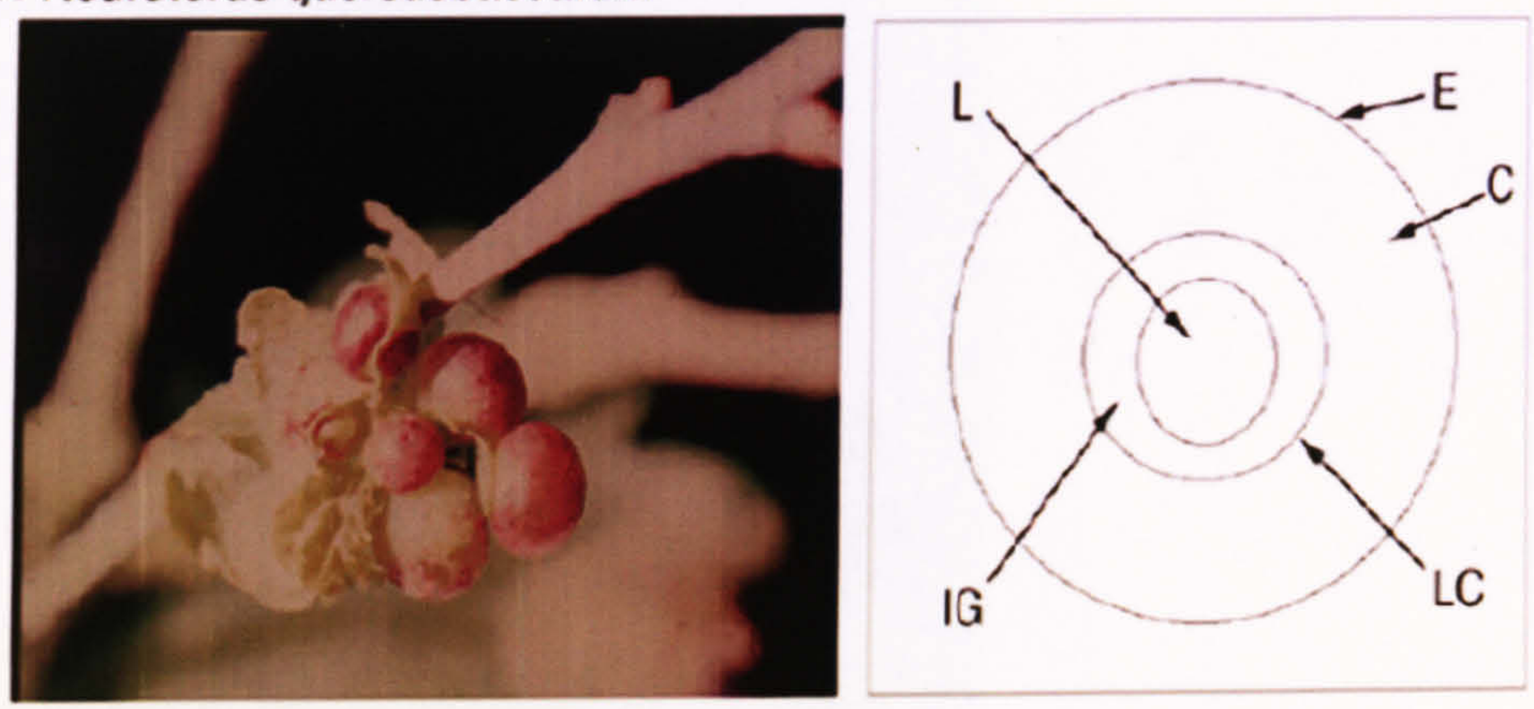
Multi-chambered leaf gall
on *Rosa canina*

C. *Andricus quercuscalicis*

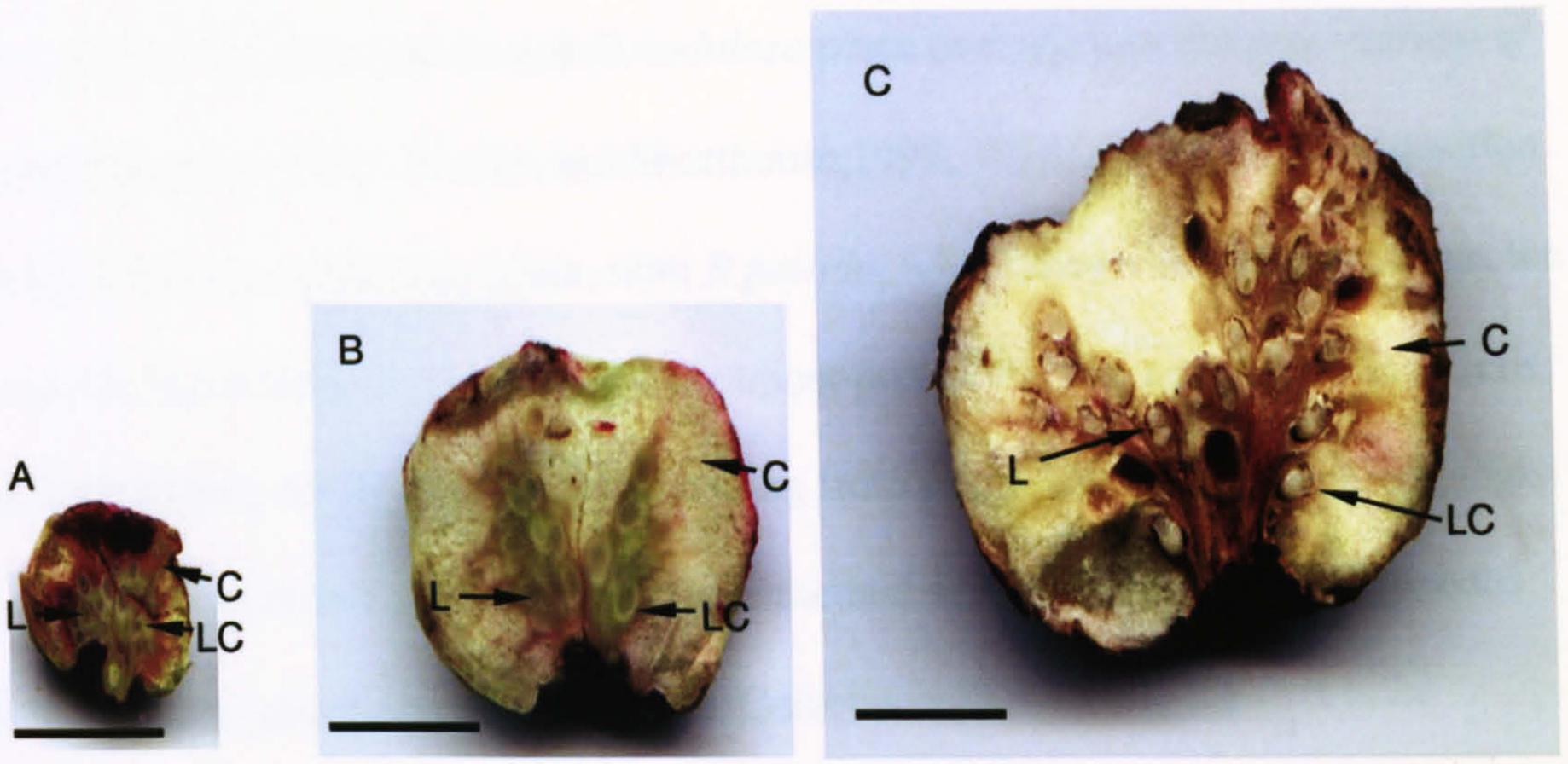


Single chambered acorn gall
on *Quercus robur*

D. *Neuroterus quercusbaccarum*



Single-chambered leaf gall
on *Quercus robur*



A. Eggs are oviposited within the buds of *Quercus robur*. Gall initiation begins and the gall soon grows out of the bud. Each larval chamber (LC) encapsulates a single larva (L). At this stage there is little inner-gall tissue lining the chamber, no schlerenchyma and a small layer of cortical parenchyma (C).
scale bar = 1cm

B. Growth by cell proliferation and cell expansion increases the size of the larval chamber (LC), the cortex (C) and the overall size of the gall. The chamber is lined with a large layer of inner-gall tissue, on which the larva graze, and a sclerenchyma layer forms around the chamber.
scale bar = 1cm

C. All the inner-gall tissue has been grazed by the larvae, and only the sclerenchyma capsule remains. The larvae start to pupate, after which they burrow out of the gall as an adult.
scale bar = 1cm

Figure 1.5 *B.pallida* at different stages of development. A) Soon after initiation. B) The growth phase. C) Maturation

To initiate galls, cynipids place their eggs onto meristematic or otherwise immortal tissues, such as cambium. Initiation begins during the process of oviposition, where wounding of the host tissue can occur, depending on the position of oviposition. Females of the agamic generation of *B. pallida*, for example, oviposit between the bud scales of *Q.robur* onto the apical meristem and *D.nodulosa* place one egg into the procambium *of R. blanda* buds (Rey, 1992; Brooks and Shorthouse,1997, 1998). During this oviposition wounding of the host tissue can occur, with *B.pallida*, where the ovipositor penetrates the young leaves, the wounded cells lyse and the upper part of the bud generally dies. Cells beneath the egg also die in response to wounding from oviposition. The cells along this lytic area dedifferentiate and proliferate within the bud scales, forming a homogeneous area, known as the gall plastem (Rey, 1992). Cells beneath these are the first to be involved in the formation of the gall and become activated showing an increase in cytoplasm, fragmented vacuoles and enlarged nuclei and nucleoli, characteristic of nutritive cells which provide nutrients for the larva (Rey, 1992; Brooks and Shorthouse, 1998). When the larva hatches, it moves into the cavity built by cell lysis beneath the egg and the regeneration around the egg (Rey, 1992; Brooks and Shorthouse, 1997, 1998).

A.glechomae places its eggs on the underside of *N.hederacea* leaves. The epidermis beneath the egg is lysed, by triggered autolysis or enzymes from the egg, enabling the egg to sink into the host tissue. Soon after oviposition, the epidermal and parenchymal cells adjacent to the egg show enlarged nucleoli and proliferate to surround the egg, in response to the *A. glechomae* egg (Taylor, 1949). Either ovipositional method securely implants the egg into the host tissue.

1.3.1.2 *Growth*

During the growth phase, the biomass of the gall vastly increases and a number of specific tissues differentiate while the gall wasp larvae themselves grow very little (Rohfritsch and Shorthouse, 1982; Rohfritsch, 1992). The most characteristic tissue is a layer of nutritive cells, common to most cynipid galls. The larva feeds exclusively on this tissue, which is continually replenished by the transformation of the nutritive parenchyma cells into nutritive cells (Bronner, 1992). In *B.pallida,* a layer of cells, like a cambium, has been observed which proliferates and replenishes the nutritive tissue towards the larva and nutritive parenchyma away from the larva (Rey, 1992). Nutritive cells are cytoplasmically dense with fragmented vacuoles and enlarged nuclei and nucleoli, and show high concentrations of lipids, ribosomes and proteins (Bronner, 1992). The cells of the nutritive parenchyma, surrounding the nutritive tissue, are less cytoplasmically dense with large vacuoles and are high in starch and low in lipids (Bronner, 1992).

The growth of *B. pallida* at this point is due to an increase in the cell size of the cambial zone within the nutritive tissue (Rey, 1992). Layers of cortical parenchyma tissue and vascular bundles form in the cortex, surrounding the inner layers. In *D. nodulaosa*, a single chambered stem gall, does not develop its own vascular system, but is surrounded by and uses the stems existing vascular system (Brooks and Shorthouse, 1998). The epidermis of all species can vary from a thin skin, as seen in *B. pallida,* to a complex tissue including stomata, hairs, spines and glands (Hough, 1953; Stone and Cook, 1998). It is these outer layers that give the mature galls their unique, species-specific character. In *A. glechomae* a cambial layer, where cells divide parallel to the epidermis, is also observed between the nutritive tissue and the parenchyma (Taylor, 1949). Vascular tissue

differentiates in the cortical parenchyma and the majority of the gall growth occurs through cell enlargement (Taylor, 1949).

1.3.1.3 *Maturation*

As the gall of *B.pallida* and *D.nodulosa* mature the cambial zone in the inner-gall disappears and the nutritive tissue is now replenished by the transformation of nutritive parenchyma into nutritive tissue (Rey, 1992; Brooks and Shorthouse, 1998). In the mature gall of *A.glechomae* the enlarged cortical parenchyma cells collapse, leaving only thin walls (Taylor, 1949). The vascular tissues and a thin layer of inner-gall tissue remain and differentiate to form a sclerenchymatous layer surrounding the larval chamber. The formation of the sclerenchymatous layer around the larval chamber is a common feature of maturation, although, not all cynipids show the presence of this layer. In the sexual generation of *Neuroterus quercusbaccarum,* for instance, this layer is not developed at any stage.

1.3.2 Inner-gall physiology

Cynipids not only control the development patterns of plants, but also the physiology of the tissues including the distribution of secondary plant metabolites such as tannins, as mentioned above. Bronner (1977,1992) has carried out extensive cytological studies, revealing a number of significant physiological gradients within the inner-gall tissue. She found that the nutritive cells have high concentrations of lipids, glucose, amino acids and high enzyme activity of acid phosphatases, glucose-6-phosphatase, proteases, amino peptidases, amylase and phosphorylase (Bronner, 1992). In contrast the nutritive parenchyma has a high concentration of starch, low concentration of lipids, glucose and

little activity of the enzymes found in the inner layers. The nutritive parenchyma cells are storage cells allowing the storage of starch, achieved by the low amylase activity. These gradients are essential for the maintenance of nutrients for the larva and together the two tissues provide nutrients for the larva's development.

1.4. Source of Morphogen

A major objective of any study of gall formation is identification of the source of the gall-inducing signals. The following sources for these morphogenetic signals have been suggested: a) a fluid deposited by the female during oviposition (i.e. the ovipositional fluid), b) the egg, or c) the larva. Here we survey briefly the evidence and arguments for these alternative sources.

1.4.1 Ovipositional fluid

During oviposition, a fluid is secreted with the egg. Little is known about the chemical compounds in this fluid in cynipids, although this fluid alone is unable to cause cynipid gall initiation. One function of the ovipositional fluid is to attach the egg to the host plant tissue. It has also been suggested that it is the transfer medium for the mutualistic viruses (Cornell, 1983). In sawflies, however, it has been demonstrated that the ovipositional fluid alone can induce gall formation (Beyerinck, 1882; McCalla *et al.*, 1962; Higton, 1994)).

1.4.2 Egg

The ovipositing female places the egg on the surface of the host plant tissue and soon after the process of gall formation begins. To look at the possible roles of enzymes in this

process, Bronner (1973) placed cynipid eggs on layers of enzyme subtrates (albumin, gelatin, cellulose and pectin) and observed the extent of substrate lysis around the egg. Using this approach they demonstrated that the eggs of *B. pallida* exude proteases, pectinases and cellulases. The simultaneous cell division seen adjacent to the egg could be caused by the presence of these enzymes, although, cell division could be the result of a wound response initiated in the plant by the lysis of host cells. The larva hatches after seven days and fragments of the egg chorion sometimes remain and provide support for the young larva while the gall continues to develop.

1.4.3 Production of morphogen in the larvae

The observation that gall formation stops when the larva is killed either by natural enemies or systemic insecticides makes it the most likely source of morphogenetic signals (Beyerinck, 1882; Rohfritsch and Shorthouse, 1982). Depending on the stage of gall formation when the gall former is killed, cells either revert to their prealtered state or remain altered. In either case, no further gall growth or development occurs (Rohfritsch and Shorthouse, 1982). This evidence suggests that the larva is providing a continuous stimulus.

Secretion of specialised compounds by the cynipid larvae might be expected to result in the characteristic cytological and morphological organisation of the larval organs. Any morphogen, secreted by the larva, would most probably be produced in one of two organs: the salivary glands or the Malpighian tubules. Rössig (1904) carried out anatomical investigations on cynipid larvae in an attempt to identify cytological and morphological differences to non-gall making insect larvae that could be interpreted as adaptations to

their gall making life-style. While the anatomical and cellular structure of the salivary gland showed no particular difference between galling and non-galling species, the Malphigian tubules of cynipid larvae showed a number of abnormalities.

1.4.3.1 Malpighian Tubules

Malpighian tubules of insects are blind-ending tubular extensions of the gut found between the midgut and the hindgut, and are responsible for the absorption of waste products from the haemolymph and their secretion into the gut. Insects can have up to 250 Malpighian tubules, although young cynipid larvae have only two exceptionally large tubules made up of fifty to sixty large cells with significantly enlarged nuclei (Rössig, 1904). In contrast to the larval state, the adult cynipid has sixteen finer and shorter tubules with normal sized cells and nuclei (Rössig, 1904). Microscope studies have shown that a fluid is continuously secreted from the Malpighian tubules, suggesting that a secretion from these may be involved in gall induction (Rössig, 1904). Further work on Malpighian tubules by Triggerson (1914) entailed injecting a saline solution of the tubules into oak leaves. The leaf tissue turned yellow- brown and began to crack in response to the extract. This response was not seen when saline solution only was injected into oak tissue, and was suggested to be the beginning of gall formation (Triggerson, 1914). The relevance to this response to gall induction remains debatable, however, and could also be caused by any number of compounds.

1.4.3.2 Salivary Glands

The salivary glands might be intuitively the most likely place for the production and

secretion of a morphogen. The larvae start to feed soon after they hatch and the feeding action of the larva would expose surrounding plant cells to the morphogen. This stimulation continues until the larva stops feeding, when the larva is ready to pupate and all the inner-gall tissue has been consumed. In other non-cynipid gall-making insects, such as coccids and aphids, gall-initiating substances have been positively identified in the saliva (Parr, 1940; Schäller, 1963, 1968; Hori, 1974, 1975,1976; Hori and Miles, 1977; Tandon and Ayra, 1980). To date, however, little work has been carried out on cynipid larval saliva and no active compound has been identified.

1.5. Putative morphogenetic signals during gall formation

As discussed above, the spatial and temporal organisation of development from a single, undifferentiated cell into tissues, and ultimately a functional organ, is generally believed to be based on morphogenetic signals. Development in general is regulated by homeotic genes, which encode DNA binding proteins that act as transcription regulators, controlling cell fate and the development of organ identity. Mutations in these homeotic genes can lead to altered cell fate, often giving rise to an organ in the wrong place or at the wrong time. Flower development, for example, can be greatly altered by mutations in the genes controlling the identity of the flower organs. It seems likely that gall-inducing insects, and particularly cynipid gall wasps, should be able to synthesize chemical compounds that are recognised by the host plant as morphogenetic signals or manipulate the concentrations and distributions of plant internal morphogenetic signals.

In gall formation, the egg is laid into meristematic cells as these are proliferating cells responsive to signals. The reprogramming of the cell fate achieved by the cynipid larva,

demonstrates that the signal is recognised by meristematic cell receptors and triggers proliferation and differentiation into a gall. There are a variety of ways by which plant growth can be altered and it remains a mystery which are required for cynipid formation. Throughout all gall formers, the active compound must interact with plant development and initiate a significant change. The specific gall morphologies could be controlled by a species-specific morphogen, or differences in spatial and temporal distribution of one common morphogen.

Studies trying to identify the means of changing plant development employed by cynipid gall wasps are few and there has been very little work carried out in the last twenty five years. As a trend, the list of putative morphogenetic compounds has grown longer the more studies have been conducted. Table 1.1 shows the proposed compounds that have been postulated as primary signals. The identity of the true morphogen remains mysterious, and for those that have been proposed, it has proven difficult to distinguish between cause and effect. The identity of the signal or signals directly responsible for the control of gall formation, therefore, remains to be determined.

Many past studies on gall induction will need to be reinterpreted in the light of current understanding of the molecular processes occurring during plant development. I will, therefore, present a historical overview of the signalling molecules proposed to be involved in gall initiation and discuss the possible role in cynipid gall induction of some classes of signal molecules recently implicated in plant development studies.

Table 1.1 Proposed Signalling molecules involved in cynipid gall formation

Proposed Signalling Molecule	Reference
RNA	Taylor (1949)
Auxins	Kaldewey (1965)
Tryptophan	Matsui (1970)
Cytokinins	Engelbrecht (1971)
Viruses	Cornell (1983)

1.5.1 Auxins

The most active of the auxins is IAA, which is responsible for many developmental processes throughout the plant. IAA promotes the growth of the main shoot whereas lateral shoot growth is inhibited (Mathews and Van Holde, 1990). It is produced from the transamination of tryptophan in the terminal bud and transported towards the shoot by the polar auxin transport mechanism (Mathews and Van Holde, 1990). Degradation of the cell wall to enable cell expansion to occur, and the induction and maintenance of vascular tissue are all controlled by IAA.

The presence of auxins have been analysed in cynipids by Kaldewey (1965), who removed the larvae of *Cynips quercusfolii* from their galls and collected their secretions in agarose blocks. He used Went's coleoptile assay and placed these blocks on one side of *Avena* coleoptiles and measured the angle of growth stimulated in cells contacting the agarose. In comparison with negative controls and blocks containing known concentrations of IAA, Kaldewey tried to quantify the amount of IAA in the cynipid secretions using the *Avena* coleoptile bioassay. Based on these experiments it was proposed that the larval secretion contained a high concentration of IAA. He also tested

the activity of homogenised larva, leaf tissue and gall tissue, finding that gall tissue was twice as active as leaf tissue, but this was still a thousand times less active than the larvae. The homogenised larva was not as active as the secretion collected from living larva, which led Kaldewey to suggest that the larvae take up tryptophan from the host tissue, transform it into IAA, and secrete it back to the host to induce growth. Although this bioassay demonstrates an IAA-like response, there is no direct evidence indicating that IAA is actually present. The assumption made by Kaldewey (1965) was that the substance that has similar effects as IAA on plant growth should at least contain this growth factor. In modern research further independent evidence such as chromotographic investigation of the larval secretion would be required. Kaldewey's experiments, however, still suggest that the larval secretion contains one or more highly concentrated compounds that promote cell proliferation. Matsui and Torikata (1970) used chromatography to study extracts of *Dryocosmus kuriphilus*. They found that IAA was not highly concentrated in either the larvae or the gall tissue. However, they did find high levels of tryptophan in *Dryocosmus kuriphilus* larva and gall tissue. Extracts from both the larva and the gall tissue induced IAA-like responses in the *Avena* coleoptile angle bioassay, as in Kaldewey's work (Matsui and Torikata, 1970). Matsui and Torikata (1970) proposed that tryptophan was active in gall formation. Tryptophan found in the nutritive cells of the inner-gall tissue, which are high in amino acids, could be taken up by the larva and converted into IAA as the larva grazes on these cells. This grazing does not occur until after the larva has hatched from the egg, when the gall has already started to form, suggesting a role for such a mechanism in gall growth rather than initiation. It might also be questionable whether the manipulation of IAA concentrations alone can result in structures as complex as cynipid galls (Weidner, 1957).

Local manipulation of IAA concentration could be brought about through manipulation of the plants own IAA transport system. IAA is transported from the apex of the plant to roots through transport cells. They enter and leave the cells by auxin-specific carrier molecules, which are found in the plasma membrane of the transporting cells (Jacobs and Gilbert, 1983; Rubery and Sheldrake, 1974; Raven, 1975). When these transmembrane carrier proteins are mutated the polar transport is disrupted and changes in plant development occur, such as a decrease in root elongation and an increase in lateral root elongation (Galweiler *et al.*, 1998).

The possible increase of IAA in galls could be attributed to an interruption of the polar transport of IAA. Apart from the changes in carrier proteins, IAA transport can also be interrupted locally by compounds such as flavonoids. Some of these compounds are known to interact with the carrier protein and thus block them for the IAA (Galweiler *et al.*, 1998). Flavonoids are present in the outer cortex of cynipid galls and their possible role will be discussed in section 1.5.5.

An alternative way to alter local IAA concentrations is based on so called IAA synergists. In a steady state IAA is continually produced and degraded. IAA synergists accelerate the function of the hormone, and have been detected in galls induced by hemiptera and their larvae (Sterling, 1952; Brian, 1957; Pilet, 1960; Hori, 1974, 1975,1976; Hori and Miles, 1977; Tandon and Ayra, 1980). The increase in IAA believed to occur in non-cynipid gall tissue could be the result of such IAA synergists. No studies that would have targeted such compounds have yet been carried out on cynipid galls or larvae.

1.5.2 Cytokinins

Cytokinins are antagonistic to auxins and together, they determine the root and shoot morphologies in normal plant development (Westhoff, 1998). Cytokinins are produced in the root and promote cell division and the elongation of cells. Lateral shoot induction and the retardation of senescence are also promoted by cytokinins. The increased cell division required for gall formation leads to the suggestion that this growth regulator could be involved in gall formation (Engelbrecht, 1971). Cytokinins and related substances have been identified in cynipid larvae and it has been suggested that they are important in cynipid gall formation (Onkaiva, 1974; Matsui and Torikata, 1970; Matsui *et al.*, 1975). Ohkawa (1974) isolated the cytokinin zeatin from *Dryocosmus kuriphilus* larvae and postulated that this is secreted from the larval salivary glands, causing gall formation by stimulating an abundance of undifferentiated cells. Ohkawa (1974) claimed that injection of zeatin into chestnut buds does induce gall formation, although unfortunately did not describe the zeatin-induced structure in detail. If these structures were to be diagnostic of the various tissues of a cynipid gall, this would have been a major breakthrough. Should zeatin, however, only induce a non-specific increase in the number of undifferentiated cells, its role in gall formation would remain undemonstrated.

Additional claims of cytokinin activity in extracts of *D. kuriphilus* larva were made by Matsui *et al.* (1975) following experiments in a callus growth bioassay. The bioassay used stem sections of chestnut, the host plant of *D. kuriphilus*, which were exposed to the larval extract and observed for callus growth. Callus growth was induced by the larval extract, and callus formation was proportional to the amount of larval extract used (Matsui

et al., 1975). An additional assay, the barley leaf chlorophyll retention test, also demonstrated cytokinin-like activity by slowing down the degradation of chlorophyll in the barley leaf (Matsui *et al.,* 1975). Although the identity of the active substance was not confirmed, thin layer paper chromatography showed that an aqueous extract of the larva contained two active components (Matsui *et al.,* 1975). Until the identity of the active components is confirmed, it is not possible to determine whether these two components are true cytokinins. The bioassays show the presence of a cytokinin-like substance, but their involvement in gall formation has not been established. Given what is known of the role of cytokinins in plant development, they are probably involved in stimulating increased cell division, and so increase in gall mass, but it seems likely that a more specific morphogen is required in addition to achieve the tissue differentiation and species specific morphologies observed in cynipid galls.

In summary, bioassays using extracts of cynipid larval salivary glands and cynipid gall tissue, have suggested that morphogens involved in gall formation generate IAA- and/or cytokinin-like responses. However, it remains unknown whether the primary signals are indeed secreted by the larva, or are instead produced by the plant as part of gall formation process and are then taken up by the larva during feeding. Such a complex reprogramming of development probably requires more than one signalling molecule, or at least a single trigger to a subsequent cascade of signalling molecules.

1.5.3 RNA

Rather than exploiting metabolically active proteins or hormones, it is also possible the

cynipids manipulate plant development by exporting RNA, which is then transcribed into proteins in plant tissues. In a study of *Aylax glechomae* on ground ivy (*Nepeta hederacea),* Taylor (1949) found significantly higher levels of RNA in larval saliva than in host meristem, mature tissues, or gall nutrituve tissue. The involvement of RNA as a signal is unlikely and the evidence on which this hypothesis is based is very weak. RNA is generally a fragile molecule, highly vulnerable to digestion by host enzymes, and so does not seem suitable as a signalling molecule passed from the larva to plant cells. A high concentration of RNA in larval saliva does not necessarily indicate a signalling role for this molecule in gall formation. Taylor, unfortunately, did not have the techniques available to identify the type of RNA in the larval saliva. No more recent assessment of the possible role of RNA in gall induction has been made.

1.5.4 Viruses

Cornell (1983) suggested that mutualistic viruses or viral particles associated with cynipids might be involved in gall formation. This is based on the observation that parasitoid relatives of the gall forming cynipids inject viruses or virus-like particles with their eggs to suppress their host's immune system. If involved in gall induction, viruses might be transferred to the host with the egg, and upon infection, induce gall formation by altering gene expression within host cells. However, the observed cessation of gall development if the larvae are killed, either within the egg or once hatched, does not support this simple virus theory. A virus may be involved, although an additional secondary signal produced from the larva would be required to maintain the infection. Cornell (1983) suggested that the salivary gland secretions maintain efficient susceptibility to the virus, while without the saliva the cells would not respond. No

viruses or viral particles have been observed in association with the cynipid or other gall-forming larva, suggesting there is no evidence to support the hypothesis. Further investigations into the presence of viral particles may reveal their presence and Cornell's (1983) hypothesis could then be considered.

1.5.5 Phenolic Compounds

Cynipid galls have long been known for their high concentrations of phenolic compounds, although their potential as signalling molecules and their potential role in gall formation have only recently been reviewed (Berland and Bernard, 1951; Hartley, 1999). Many defence products such as tannins, flavonoids and other phenolic compounds, are known to be found in high concentration in the cortex of cynipid galls and are believed to have a role in fungal defence and also help protect against herbivorous attack (Taper and Case, 1987; Berland and Bernard, 1951). These plant secondary metabolites are involved in general plant defence processes, induced by pathogen attack or the wounding of plants (K·c, 1997). Wounding does not always occur during oviposition, although the enzymes exuded by the egg which cause cell lysis may induce a wounding response, through the phenylpropanoid pathway (Hahlbrock *et al.*, 1981).

Phenolic compounds are also known to affect plant growth, especially of meristematic tissue (Kefeli and Kutacek, 1976). Phenolic acid, for example, is a natural protector of IAA, preventing its degradation by peroxidase and so prolonging its effect (Kefeli and Dashek, 1984). Flavonoids are known to block polar transport and therefore cause an accumulation of auxin within tissues, which changes the direction of growth, as previously discussed (see section 1.5.1). Thus, the increase in IAA concentration and the rate of cell

division observed in galls could also be in response to flavonoids that are induced in the host as a defence response and accumulate in the outer cortex of the gall. This would be analogous to the response of legumes to the bacteria symbiont *Rhizobium* (see Figure 1.6 for process of nodulation) in which the host's flavonoids cause a significant increase in the concentration of IAA within the nodules housing the symbiont (Danger and Basu, 1987; Prinsen *et al.*, 1991; Mathesius, 2001). It has been demonstrated that nodule-like structures can be induced on legumes after treatment with two known auxin transport inhibitors, N-1-naphthyl phthalamic acid and 2,3,5-triodobenzoic acid (both phenolic compounds) (Hirsch *et al.*, 1989) suggesting that natural nodules could be induced in the same way. Genes expressed early on in response to *Rhizobium* infection known as Early nodulin genes (ENOD) were also expressed within these artificial nodules (Hirsch *et al.*, 1989). An accumulation of auxin is also seen in the galls formed by the root knot nematode, and this too has been explained by the flavonoid pathway (Hutangura *et al.*, 1999).

1.5.6 Oligosaccharides

Oligosaccharides are one of four compounds newly-identified as plant growth regulators, jasmonates, brassinosteroids, and peptide hormones being the other three (Westhoff, 1998). Although oligosaccharides have not previously been investigated as potential morphogenetic signals in cynipid gall formation, recent studies in plant development suggest a wide scope for signal transduction pathways that involve different kinds of oligosaccharides as signals. Below, several of the main classes of oligosaccharide plant-signals are discussed. Some have known impacts in other galling

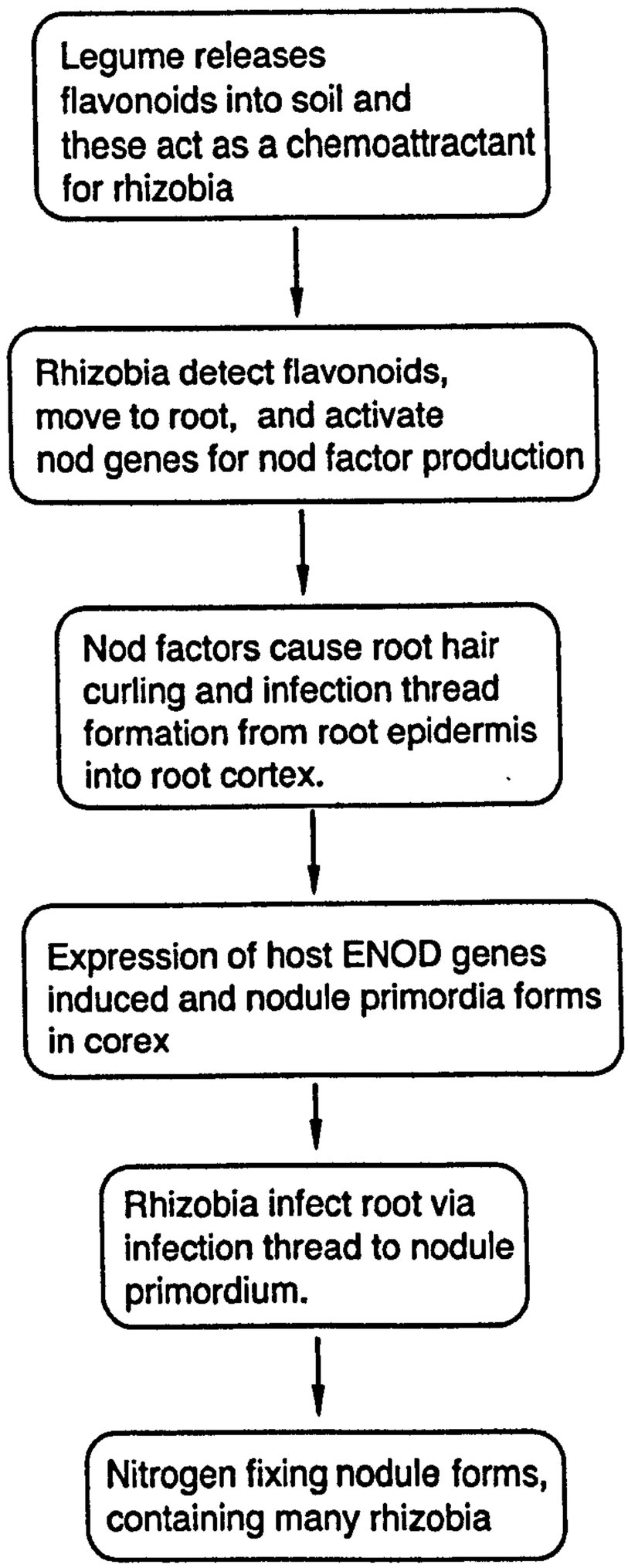


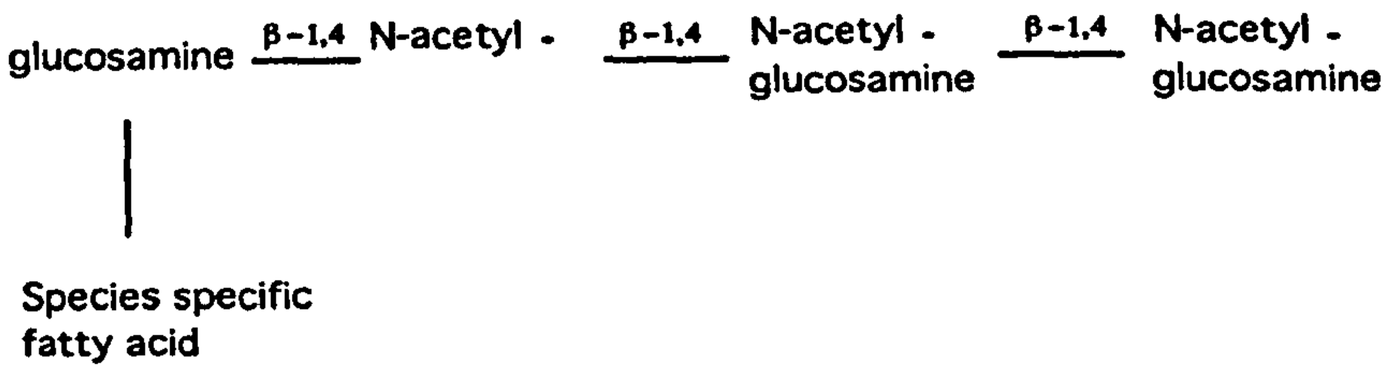
Figure 1.6 The steps involved in nodulation by *Rhizobia*.

systems, particularly *Rhizobium* root nodule galls in legumes. None yet has a demonstrated role in cynipid galls.

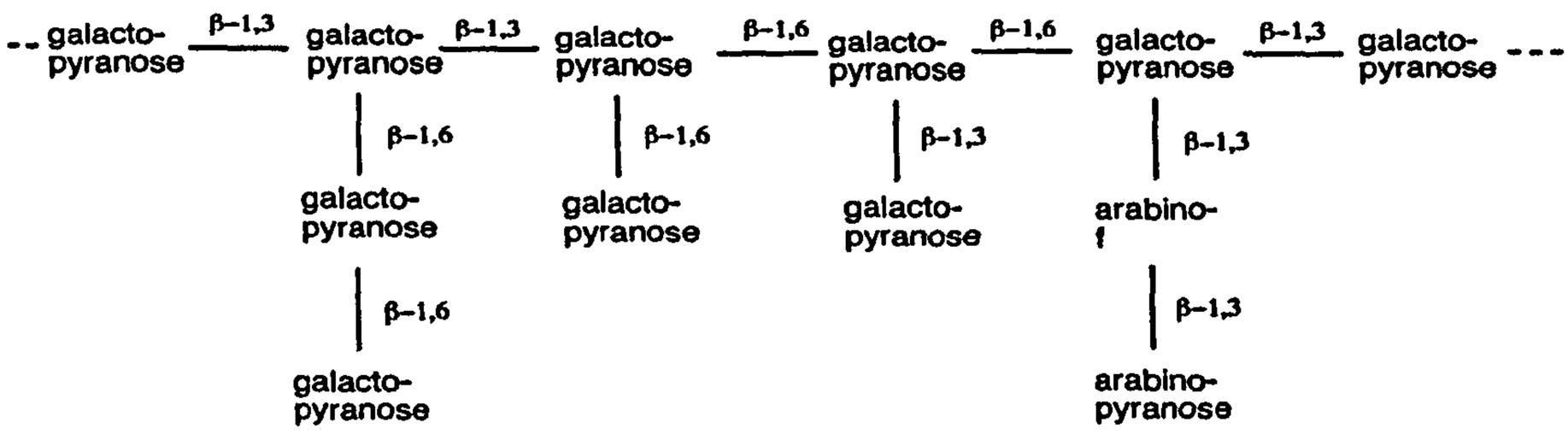
(a) Lipo-chitooligosaccharides, or Nod factors, control the formation of the nitrogen-fixing root nodules in the symbiotic *Rhizobium*-legume relationship (see Figure 1.6). They are known to trigger cell proliferation and the expression of the host's early nodulin genes (ENOD), all are discussed in detail in chapter 3. The Nod factors, composed of a chitooligosaccharide backbone (see Figure 1.7a), are produced by the *Rhizobium* with species-specific side chains, achieving specificity to a narrow range of hosts for each strain of *Rhizobium*. Nod factors have been shown to be active in other plant systems, activating arrested somatic embryos and increasing cell proliferation, demonstrating a role in plant growth and organ development (De Jong *et al.,* 1993; Röhrig *et al.* 1995).

(b) Arabinogalactan proteins (AGPs) are high molecular weight proteoglycans with less than 10% protein but high levels of arabinosyl and galactosyl residues (Schulz *et al.,* 1998)(see Figure 1.7b). They can be constituents of plant cell walls or secreted by cells and are believed to have a role in cell-cell signalling, cell division, differentiation and somatic embryogenesis (Knox, 1995). They can be found in leaf, stem, seed, root and floral tissue and throughout the plant kingdom, although majority of the work has been carried out on angiosperms (Jermyn and Yeow, 1972; Clarke *et al.,* 1979; Fincher *et al.,* 1983). They are found at the plasma membrane or in cell walls and are secreted in sap and gum and are secreted in cell suspension cultures. They are defined in two groups: type I and type II (Aspinall and Cotterell, 1971). Type I have a linear (1-4)-ß-D-galactan backbone and arabinose

a) Lipo-chitooliosaccharides (Nod Factors)



b) Arabinogalactan protein of larch *(Larix laricina)*



(Partial structure)

c) Xyloglucan nonasaccharide

d) Oligogalacturonides

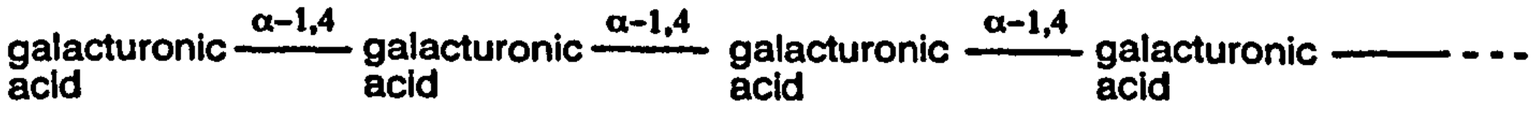


Figure 1.7 The structure of oligosaccharides a) lipo-chitooligosacharide. b) Arabinogalactan protein. c) Xyloglucan nonasaccharide. d) Oligogalacturonides.

oligosaccharide side chains. Type II have a highly branched (1-3)-ß-D-galactan backbone with (1-6)-ß-D-galactan sidechains. A solution known as Yariv reagent or Yariv phenylglycosides, binds to and precipitates AGPs producing a red precipitate (Yariv *et al.*, 1962). Using Yariv reagent it has been demonstrated that the removal of AGPs from cell suspension media arrests cell division, which can be restored by transferral to medium not treated with Yariv regent (Serpe and Nothnagel, 1994). Demonstrating, therefore, that AGPs secreted into the media are necessary for cell-cell communication.

Recent evidence shows that immature and secreted AGPs may in fact be the substrate of endochitinases in embryogenesis and together produce a signal involved in embryogenesis (Van Hengel, 2001). Analysis of immature seed AGPs and those secreted from cell cultures using thin layer chromatography, gas liquid chromatography and gas chromatography-mass spectrometry, demonstrated the presence of oligomers of N-acetyl-D-glcosaminyl and glucosamine (GlcNAc) (Van Hengel, 2001). These AGPs are sensitive to endochitinases, hydrolyzing the (1-4) link between 3 or more GlcNAc. They can restore arrested somatic embryogenesis and chitinase treated AGPs even showed an increase in embryogenesis, demonstrating that, depending on their surrounding, AGPs may be able to produce a number of signals controlling cell division and cell fate (Van Hengel, 2001). The widespread signalling properties of oligosaccharides are also demonstrated.

AGP spatial and temporal expression can be used as markers of cell types and as an indication of the eventual fate of specific cells. This is shown by JIM 13, an antibody

to a plasma membrane AGP, which can be detected in cells which are to form xylem, before xylem differentiation is observed in radish, carrot and pea (Knox *et al.*, 1991; Casero *et al.*, 1998). Another AGP antibody, JIM 8, binds to an AGP indicative of cells able to undergo somatic embryogenesis (McCabe *et al.*, 1997). Cells which are JIM 8 negative do not form somatic embryos, however, can be induced to do so if exposed to conditioned medium in which JIM 8 positive cells have been cultured, thus showing the presence of a soluble signal which, from GC/MS analysis, is likely to be the JIM 8 AGP. This signal is thought to be important during the early stages of somatic embryo development as it becomes inhibitory in more mature embryos (McCabe *et al.*, 1997). Such signals could be used for cell-cell communication during gall formation and the expression of particular AGPs on certain gall cells could be indicative of cell identity or eventual cell fate, which will be discussed further in chapter 5.

(c) Xyloglucan

Another component of plant primary cell walls is xyloglucan, which cross links the cellulose microfibrils making up the wall (Cutillas-Iturralde and Lorences, 1997). Xyloglucan is associated with cell wall loosening and it is believed that the hydrolysis of xyloglucan by such enzymes as cellulase enables cell expansion (Cutillas-Iturralde and Lorences, 1997). Xyloglucan can be broken down into oligosaccharides which, depending on their composition, can cause a range of responses in the plant. Xyloglucan-derived hepta(7) and octa(8) saccharides are known to elicit a general defence response within the plant (Pavlova *et al.*, 1996). More detailed investigations have revealed that a nona(9) saccharide with a terminal fucosyl residue (see Figure 1.7c) inhibits the effects of auxin

promoted growth in the plant. The reduced octasaccharide had the opposite effect and promoted an auxin-like growth response by increasing a-L-fucosidase activity, in the absence of auxin (Cutillas-Iturralde and Lorences, 1997). In relation to gall formation the xyloglucan-derived oligomers with the auxin-like growth response may be released from the host plant cell wall by hydrolytic enzymes from the egg or larvae. These would loosen the cell wall and cause cell growth.

(d) Pectin

Pectin is found within the cell wall and can be broken down by pectin lyases and polygalacturonidase to release oligogalacturonides (see Figure 1.7d), which have been described as signal molecules involved in plant defense responses. Oligomers of 10-20 residues show biological activity and induce a localised defense response causing the production of many defense related compounds including phenylalanine ammonia lyase, chitinases and glucanases (John *et al.*, 1997). Other signalling properties of oligogalacturonide include the inhibition of root growth on tobacco explants, cuttings from a plant grown in culture (John *et al.*, 1997). This inhibition can be overcome by the application of auxin demonstrating an antagonistic relationship (John *et al.*, 1997). Pectin lyases and polygalacturonidases, required to release the oligogalacturonides from the cell wall, have been identified on cynipid eggs and are also probably contained within the larval saliva (Bronner, 1973, 1977). Bronner (1973) demonstrated that the egg secretes pectinases and it is known that the larval saliva contains active enzymes. The activity of these enzymes would result in a thinning of the cell wall. This is consistent with the reduction of pectin which has been observed in host plant tissues within the first day of gall formation (Rohfritsch and Shorthouse, 1982). The reduction of pectin increases the

permeability of the cell wall. This would enable larger signal molecules, previously impermeable to the cell, to enter the cell, and perhaps initiate gall formation.

In summary, several types of oligosaccharides elicit a wide range of responses, either known or thought to be important in gall formation. Relatively simple modifications of molecular structure can modify such responses. Signal transduction pathways involving oligosaccharides have been shown to work in a taxonomically diverse range of plants. Finally, oligosaccharide molecules might provide the diversity of gall structures we might expect from the signal.

1.6 Modern Techniques

Today, with modern molecular techniques, it should be possible to identify genes or proteins that are expressed differentially during the formation of galls. Studies on differential gene expression and specifically expressed proteins in gall tissues should indicate the pathways involved in gall formation and provide markers to be used in a bioassay. Molecular markers expressed early in gall development also have the added advantage that it would not be necessary to wait for tissue differentiation and the formation of a gall. Bioassays used in the past do not meet these requirements and their test for a functional response. For example the *Avena* assay, is ambiguous as to whether effects are caused by primary or secondary signals and as to whether this tested response is actually involved in the gall formation process. With improved molecular techniques we now have the tools to elucidate the mechanism of gall formation and a more comprehensive understanding of plant development. With the identification of gall proteins and their expression patterns throughout gall development, in particular gall

induction, it will allow the design of unambiguous bioassays, not only for gall induction, but for all the different phases of gall formation and the signals involved to be elucidated. Such bioassays together with improved analytical methods such as gas chromatography-mass spectrometry (GC/MS) and high pressure liquid chromatography (HPLC), should allow to unravel the secret of gall formers: how to talk to plants.

With this in mind, the aim of my research was to analyse cynipid gall formation in an attempt to further our understanding of the molecular mechanisms involved in the interaction and identify possible markers for the development of a gall formation bioassay. I took four approaches:

1) A PCR based approach to search for genes to known signalling molecules, Nod factors, within the gall wasp genome. Nod factors are chitinoligosaccharides and are used by Rhizobia in the Rhizobia-legume interaction, to induce nodule formation in the host plant. These signals are formed from bacterial *nod* genes, and it has been suggested that Nod factors may be used as a widespread signalling molecule during development. To investigate the presence of *nod* genes in the cynipid gall wasp genome, PCR analysis was carried out to look for *nodC* homologous sequences, and is presented in chapter 3.

2) Protein analysis of the inner-gall tissue was carried out to investigate the protein signatures of inner-gall tissue and non-gall tissue and is presented in chapter 4. SDS-PAGE, Coomasie Brilliant blue staining and western analysis were used to identify inner-gall proteins and analysethe variation in protein content between different gall species and to non-gall tissue.

3) Cytological analysis of the inner-gall tissue throughout the development of several gall species is presented in chapter 5. Tissue sections were used to investigate the cytological

changes throughout development in several gall speicies. Immunohistochemistry was used to analyse the spacial distribution of an inner-gall protein and cytological features of the inner-gall tissue.

4) The development of a gall formation bioassay to enable the activity of possible signals involved in gall formation was initiated and is discussed in chapter 6. Rose callus tissue was used as a test tissue and the cynipid larval extract was exposed to this as a source of the active molecules. The induction of proteins in the callus after exposure to the larval extract was used as a molecular marker for activity.

2: Materials and Methods

2.1 Materials

2.1.1 Living material

Diplolepis spinosa L. (Hymenoptera: Cynipidae), a Canadian multi-chambered stem gall which forms on the meadow rose and has one generation a year. Material for this was kindly provided by Professor Joe Shorthouse, Laurentian University, Ontario, Canada as part of a collaboration.

Diplolepis rosae L. (Hymenoptera: Cynipidae) which has only one generation each year and forms hairy, multilocular galls on the buds, leaves or stem of *Rosa* species.

Biorhiza pallida L. (Hymenoptera: Cynipidae) also known as the "oak apple", has two generations a year forming on *Quercus robur*. The bisexual generation which forms in spring as a multilocular bud gall on *Q.robur* was used and collected once or twice weekly between the end of April to June from local English woodland and London parks.

Neuroterus quercusbaccarum L. (Hymenoptera: Cynipidae) has two generations, a spring and an autumn gall. The spring bisexual generation was used for this investigation and forms as a monolocular, green, spherical gall (5-7mm) on the axis of the catkins or on the underside of the leaves. Collections were made once or twice weekly from April to May from local English woodland and London parks.

Cynips quercusfolii L. (Hymenoptera: Cynipidae) has two generations a year, the bisexual generation forms unnoticeable bud galls in spring. The unisexual generation, like *N.quercusbaccarum,* is a monolocular, pale green, spherical gall (15-25mm), forming on the underside of *Q.robur* leaves.

Andricus quercuscalicis L. (Hymenoptera: Cynipidae) has two generations each year. The agamic generation was used in this investigation, a single chambered gall formed in the developing acorn, also known as the "knopper gall". Collections were made once or twice

weekly from July to the end of August from local English woodland and London parks.

Andircus fecundator L. (Hymenoptera: Cynipidae) has two generations a year and the autumn, unisexual generation was used in this investigations, which forms monolocular bud galls on *Q.robur*. Collections were made once or twice weekly from July to the end of August from local English woodland and London parks.

Quercus robur L. leaves, stems and acorns from Mile End Park were used for protein extraction, in the bioassay and for callus formation.

Rosa rugosa L. leaves, stem and seeds were collected from bushes around Mile End Park and used for protein extraction, in the bioassay and for callus formation.

Profonuca pygmea L. was collected form *Q.robur* trees in Mile End Park.

Pisum sativum L. Onward (Main crop) from Thompson and Morgan, Ipswich, England.

2.1.2 Consumables

Low melting point agarose, DNA molecular weight markers, dNTP's, restriction endonucleases were purchased from Helena biosciences, Sunderland, Tyne on Wear.

Ampicilin, kanamycin, salmon sperm DNA, Ficoll, sucrose, RNase A, ß-merccaptoethanol, proteinase K, DTT, phytagel, BAP, MS basal media, TEMED, 2-4-D, Tween 20, Trizma base, streptavidin-peroxidase, biotin protein molecular weight marker, prestained protein molecular weight marker, rainbow protein molecular weight marker, Coomassie Brilliant Blue R, casein were all purchased from Sigma Aldrich Company Ltd. Poole, Dorset.

IPTG and X-gal were purchased from Melford labs.

Acrylamide (40% w/v 29:1:0.9), sterile disposable plasticware (petri dishes, 15ml and 50ml tubes), 1.5ml and 2ml eppendorf tubes, superfrost microscope slides,1ml and 2.5ml sterile syringes, glycine, glycerol (AnalR), NaCl, EDTA , ethidium bromide, were all purchased from Merck Ltd. Lutterworth, Leicestershire.

T4 DNA ligase and ligase buffer were purchased from Gibco.

3mm chromatography paper was purchased from Whatman.

Hybond N^{TM}, Hybond C^{TM}, ECL detection kit, Ready to go DNA labelling kit, ConA sepharose were all purchased from Amersham Pharmacia Biotech, Buckinghamshire

Microcon-30 and Microcon-100 spin columns were purchased from Millipore UK Ltd. Watford.

Biorad protein assay and thick blotting filter paper were purchased from Bio Rad laboratories Ltd, Hemel Hempstead.

0.2µm syringe filters were purchased from Sartorious Ltd. Epsom, Surrey.

Plasmid DNA purification midi kit, gel extraction kit and QIA quick PCR purification kit were purchased from Qiagen, Crawley, West Sussex.

pGEM™-T easy cloning vector was purchased from Promega.

Vecta bond reagent and mounting medium containing 10% DAPI was purchased from Vector laboratories Ltd. Peterborough.

Tissue-tek (O.C.T 4583) compound was purchased from Agar Scientific, Stanstead, Essex.

Oligonucleotides were purchased from Genosys, Cambridge.

DNA detection kit, NBT/BCIP detection reagent from Life Technologies,

Anti-digoxigenin conjugated to alkaline phosphatase, blocking reagent from Boehringer Manheim, Lewes, East Sussex.

2.1.3 Plasmids used

A plasmid, pKT230, containing a 6.6kb sequence of *nodEFDABC* genes was provided by Alan Downie from The John Innes Centre, Norwich (Rossen *et al.*, 1984). I named the plasmid pC98 and it is shown in Figure 2.1.

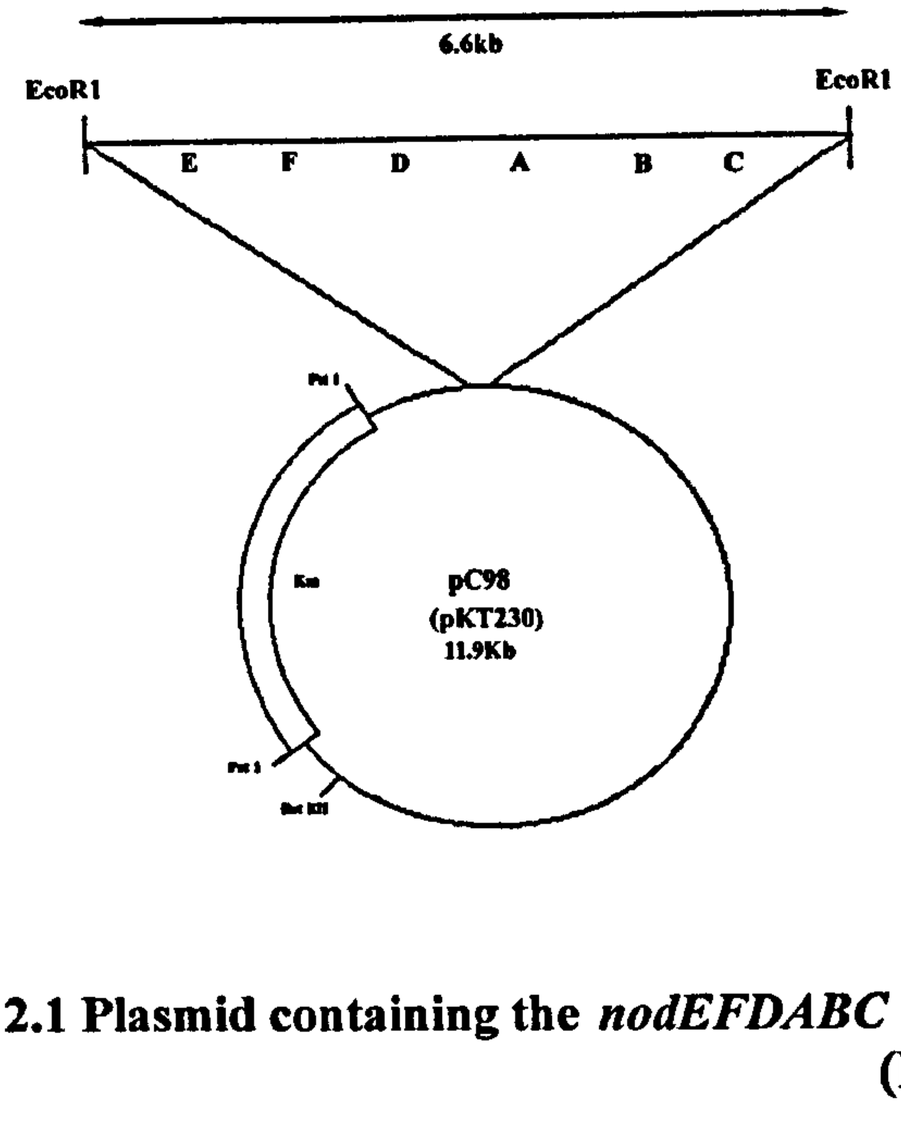


Figure 2.1 Plasmid containing the *nodEFDABC* genes (Rossen et al., 1984)

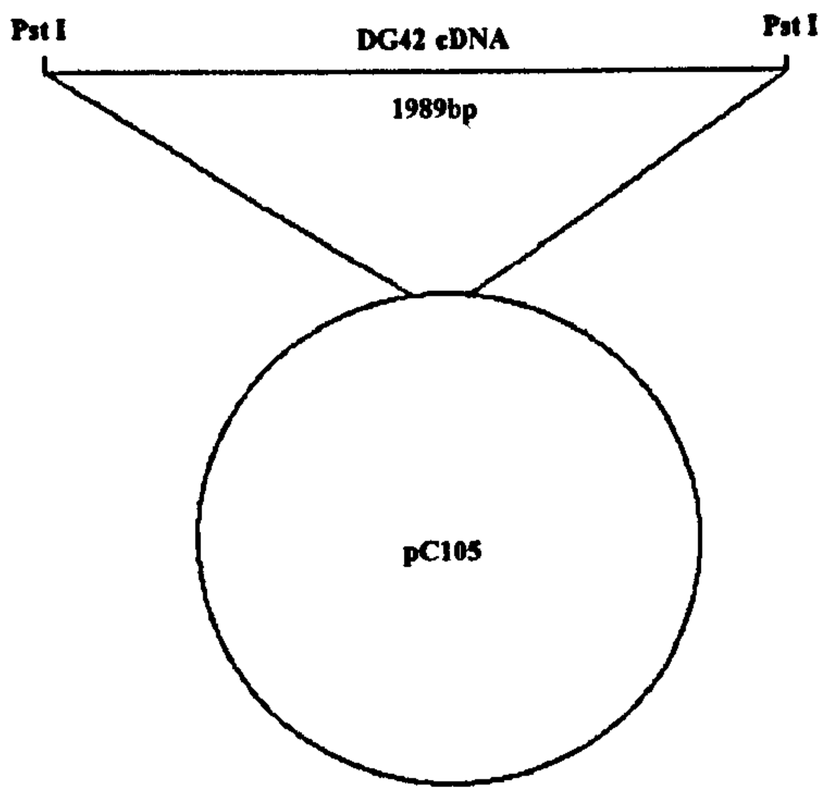


Figure 2.2 Plasmid containing DG42 cDNA (Rossa et el., 1988).

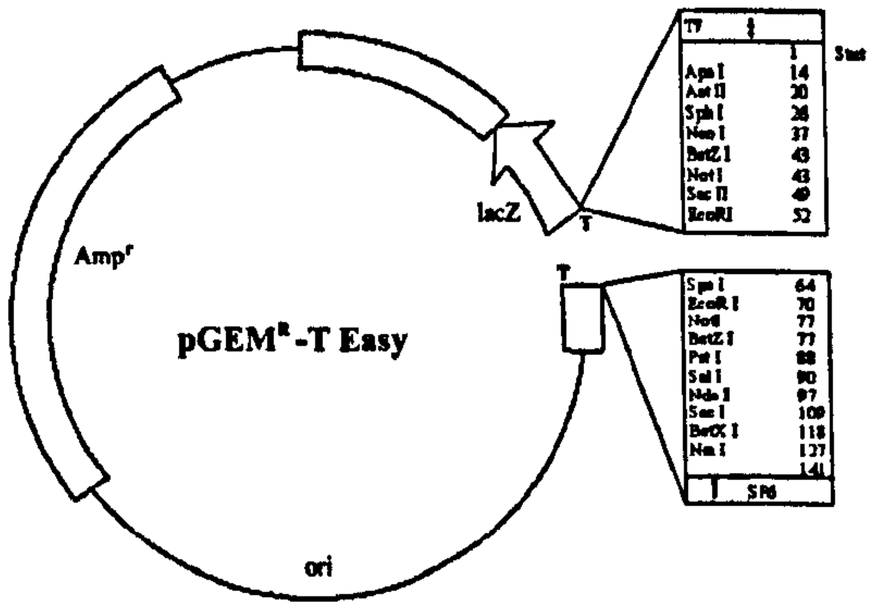


Figure 2.3 pGEM‘ -T-easy vector used to clone the PCR product.

A plasmid, pC4202, containing the DG42 cDNA was provided by Prof. Igor Dawid from the Laboratory of Molecular Genetics, National Institute of Health, Maryland (Rosa *et al.*, 1988). I named the plasmid pC105 and it is shown in Figure 2.2.

The pGem™-T-easy cloning vector from Promega was used and is shown in Figure 2.3.

2.1.4 Equipment

A PTC-200 Peltier Thermal cycler was purchased from GRI.

Submarine DNA electrophoresis apparatus was purchased from Hoefer. A minigel system from BioRad was purchased for SDS-PAGE of protein sample and a semi-dry electroblotter for blotting proteins.

2.1.5 Medium

Luria Broth was made using 10g tryptone, 5g yeast extract, 5g sodium chloride dissolved in 1 litre of distilled water and adjusted to pH 7.5 with sodium hydroxide before autoclaving. Solid medium for plates was made by adding 1.5% agar to the LB prior to autoclaving. LB-ampicinlin and LB-kanamycin plates were made by adding 50µg/ml of the appropriate antibiotic to the molten LB-agar after autoclaving before pouring. When blue/white screen was required 120µg/ml IPTG and 40µg/ml X-gal were added before pouring the plates.

Murashuge and Skoog basal media: 1x MS mix, 3% sucrose, 200mg casein, 500ng 2,4-D (dichlorophenoxyacetic acid) made up to 1 L in distilled water and autoclaved. 600µl of 1mg/ml BAP (6-benzylaminopurine) was added to the MS media immediately before pouring. When solid media was required 2g phytagel was added prior to autoclaving.

2.2 Methods

2.2.1 PCR techniques

2.2.1.1 Purification and Extraction of template DNA

Three different template DNA samples are required for the PCR: DG42 *nodC* and cynipid gall wasp genomic DNA.

2.2.1.1 (i) Purification of *nodC* DNA

A glycerol of *E.coli* transformed with the plasmid containing *nod* genes was sent from Alan Downie, shown in Figure 2.1. This was streaked out on LB-kanamycin agar plates for single colonies and one of these was picked and grown to stationary phase in 100ml liquid cultures of pC98 in LB kanamycin overnight on a rotary platform at 37°C. To pellet the cells the cultures were centrifuged for 30 min at 6000g at 4°C. The pellet was resuspended in 4ml TSE (25% w/v sucrose, 5mM Tris-HCl pH 8.0 and 50mM EDTA) 0.5ml of lysosyme solution (10mg/ml) and incubated on ice for 10 min. 5ml of lytic mixture (2% Triton x-100, 50mM Tris HCl pH8.0 and 50mM EDTA) was added and left at room temperature to lyse the cells. This was centrifuged for 60 min at 6000g 4°C, pelleting bacterial debris and chromosomal DNA. The supernatant, containing the plasmid DNA was transferred to a clean tube and the DNA was purified by ethanol precipitation. 0.1 volume of 3M sodium acetate pH4.8 and 2.5 volumes ofabsolute ethanol was added to the supernatant. This was left for 30 min on ice and centrifuged for 30 min. The supernatant was discarded and, to wash the pellet, 70% ethanol was added to the pellet, vortexed and spun down for 5 min. Three washes were carried out in total and after the final wash the supernatant was removed and the pellet left to air dry at room

temperature. The pellet was resuspended in 100µl TE (10mM Tris HCl pH 7.6, 1mM EDTA pH 8.0).

2.2.1.1 (ii) DG42 cDNA

The DG42 DNA was provided by Prof. Egor Dawid as an aliquot of plasmid containing the cDNA DG42 sequence which could be used without preparation in the PCR reaction.

2.2.1.1 (iii) Extraction of cynipid gemonic DNA

The extraction protocol recommended by Dr James Cook (unpublished data) was carried out to obtain cynipid genomic DNA. Freshly dissected *Andricus quercuscalicis* larvae were used and after dissection from the gall, the guts of the larva were removed and a single larva placed in a microcentrifuge tube. 500µl of TNE (50µM Tris pH 7.5, 100µM NaCl and 10µM EDTA pH 8.0) and 20µl proteinase K (20mg/ml) were added. The larvae were homogenised using a plastic pestle and incubated at 37°C for 3 h. This was centrifuged in a microfuge for 5 min and the supernatant transferred to a clean microcentrifuge tube. 170µl of 5M NaCl was added and, after shaking by hand, centrifuged for 5 min. The supernatant was transferred to a clean tube and centrifuged for 5 min and transferred again to a clean tube. 800µl 95% ethanol was added and tubes placed at –20°C for 30 min and centrifuged for 30 min. The supernatant was discarded and the remaining pellet washed in 70% ethanol and left to air dry. The pellets were resuspended in 50µl TE (10mM Tris HCl pH 7.6, 1mM EDTA pH 8.0) and 1µl was run on a 0.8% agarose gel (section 2.2.1.2) to verify the extract.

2.2.1.2 DNA gel electrophoresis

All DNA gel electrophoresis was carried out using 0.8%-1.7% agarose gels with 0.5 x TBE running buffer in horizontal submarine minigel tanks at a constant voltage between 20 and100 V. The agarose powder (0.8%-1.2% w/v) was mixed with 100ml of 0.5 x TBE running buffer and heated for 2 min in a microwave until melted. 0.5µg/ml ethidium bromide was added to the agarose before pouring into the gel apparatus and allowing to set. Up to 25µl sample were run in each well, mixed with 6 x loading dye (6 x TBE, 60mM EDTA pH8, 0.6% SDS, 1.5g Ficoll 400, 25mg bromophenol blue made up to 10ml with distiled water). After electrophoresis, the gels were viewed under a short wave (260nm) transilluminator and photographed using a UVP video system attached to a video copy processor. When the samples were to be recovered from the gel, a long wave (365nm) transillumnator was used.

2.2.1.3 Molecular weight markers.

1kb or 100bp size markers from Helena Bioscience were run with samples, and when quantification of DNA samples was required, a HindIII digested lambda DNA ladder was used. The known quantities in each band enables quantification of DNA in samples.

2.2.1.4 Design of oligonucleotides

2.2.1.4 (i) NodC primers

The consensus sequence of NodC from the alignment of several rhizobia was used in the creation of degenerate primers shown below. Conserved regions were chosen from the 5'

and 3' end of the sequence and, as nested PCR was to be carried out, an outer and an inner primer were created at each end.

nodC 5'outer: TAYGTNGTNGAYGAYGGN,

nodC 3'outer: NGCCCANCKNARYTGYTG,

nodC 5'inner: AAYGTNGAYWSNGAYACN,

nodC 3'inner: NGGNCCRCARCARCACAT.

(Key: R = A+T, Y = C+T, M = A+C, K = G+T, S = G+C, W = A+T, H = A+T+C, B = G+T+C, N = A+G+C+T, V = G+A+C).

2.2.1.4 (ii) DG42 primers

To create the DG42 primers the consensus sequence from the alignment of NodC, DG42 and other Nod C homologues was used. A set of 4 primer were chosen: 5' outer and inner primers and 3' outer and inner primers shown below.

DG42 5'outer: CARGTNTGYGAYWSNGAY,

DG42 3'outer: CCANCKNGTYTGYTGRTT,

DG42 5'inner: GARATGGTNAARGTNYTNGAR,

DG42 3'inner: RTTNGTNARRTGNCTRTCRTC

(Key: R = A+T, Y = C+T, M = A+C, K = G+T, S = G+C, W = A+T, H = A+T+C, B = G+T+C, N = A+G+C+T, V = G+A+C).

2.2.1.5 The PCR reaction mixture

The mix was a total of 50µl containing: 5µl of 10 x PCR buffer (100mM Tris-HCL pH8.3

500mM KCl), 0.5µl dNTPs from a 25mM stock, 0.5µl $MgCl_2$ from a 100mM stock solution, 1µl Taq polymerase from a 5 units /ml stock solution, 1.25µl of each primer from a 10mM stock solution, 1µl of DNA template and the volume was made up to 50µl with sterile distilled water. This was made up and carried out in 200µl thin walled PCR tubes.

The usual combinations of primer and template were: *NodC* primers on *nodC* template (control), *NodC* primers on *Xenopus* template, DG42 primers on *Xenopus* template (control), DG42 primers on *nodC* template, *nodC* primers on *A.quercuscalisis* template, DG42 primers on *A.quercuscalicis* template. The PCR products were run on a 1.7% TBE gel (see section 2.2.1.2) along side a 1kb or 100bp size ladder and observed on a shortwave tranilluminator and photographed.

2.2.1.6 PCR programme

Many PCR programmes were used during optimisation. The final PCR programme was a split programme with 1 cycle at 94°C for 4 min, 5 cycles with: 1min at 94°C, 1min at 42°C and 1min at 72°C, and 35 cycles with: 1min at 94°C, 1min at 48°C, 1min at 72°C. A final stage of 1 cycle at 72°C for 10 min.

2.2.2 Analysis of DNA by hybridisation

2.2.2.1 Southern blot transfer

To identify specific fragments of DNA, Southern blot analysis was carried out, as described by Southern (1975). DNA samples or PCR reactions were run on 1.2% TBE agarose gels (see section 2.2.1.2) and treated to prepare for Southern blotting. The gel was

first rinsed in distilled water and soaked in denaturation buffer (1.5M NaCl, 0.5M NaOH) with gentle shaking for 15 min. The solution was poured off and the gel rinsed with distilled water before soaking in denaturation buffer with gentle shaking for 15 min. The gel was rinsed again and placed in neutralization buffer (1.5M NaCl, 0.5M Tris-HCl pH 7.2, 0.1M EDTA) with gentle shaking for 15 min, rinsed and placed in neutralization buffer for a further 15 min. The capillary blots was then set up to transfer the DNA onto a nylon filter (Hybond-N). This entailed placing the gel on a platform with 3 sheets of 3MM filter paper cut to the same width as the gel and long enough to reach the reservoir of transfer buffer 20 x SSC (3M NaCl and 0.3M sodium citrate) under the platform. Hybond - N membrane was cut to the same size as the gel and placed on the gel, three more sheets of filter paper were placed on top of this followed by a stack of paper towels. A 0.75 kg weight was placed on top of each blot and left overnight. Once transferred, the membrane was then carefully removed from the apparatus and left to dry in the dark before being cross-linked in the crosslinker for 120 sec.

2.2.2.2 Purification of probe DNA

To purify the DG42 fragment from the plasmid to use as a probe in the Southern analysis, an endonuclease restriction digest using Pst 1 was set up. A 40µl reaction containing 35µl pC105 plasmid DNA, 1µl Pstl (10units/µl) and 4µl of the appropriate 10 x buffer were incubated for 2 h at 37°C. The entire digest was loaded on a 0.8% agarose gel and after electrophoresis the 1694bp DG42 fragment was cut out of the gel with a scalpal and purified using Geneclean spin filters. This involved placing the eluted band into a Wizard minicolumn and centrifuging for 5 min. The DNA was then precipitated from the supernatant by ethanol precipitation. This involved adding 0.1 volumes of NaAc pH 4.8

and 2.5 volumes of ethanol to the product of the spinning purification. This was left in a -20_C freezer for 30 min and then spun for 30 min in a 4°C microcentrifuge. The supernatant was removed, 1ml of 70% ethanol added and spun for 10 min. The supernatant was removed and the pellet was resuspended in 20µl of TE.

2.2.2.3 Labelling of DG42 cDNA

The probe was prepared using Ready to Go DNA labelling kit from Amesham Pharmacia biotech, which uses random primed synthesis incorporating the ^{32}P dCTP to label the DNA. The Ready to Go was reconstituted with 20µl dH_20 and placed on ice for 30 min. Purified pC105 fragment (see section 2.2.2.2) containing the DG42 sequence was made up to 50ng/25µl with TE, this was denatured for 5 min at 94°C and placed on ice for 2 min then microfuged before adding to the Ready to Go. 5µl (50µCi) ^{32}P dCTP was added to the Ready to Go in the hot lab and pipette up and down, then left at 37°C for 15 min. A 1ml syringe barrel was filled with sephadex put inside a 10ml falcon tube and centrifuged at level 4 for 2 min. A microfuge tube without a lid was placed in the bottom of the falcon tube under the syringe. The probe from the Ready to Go tube was then placed in the syringe on top of the sephadex and centrifuged at level 4 for 2 min. The probe which had passed through the sephadex was pippetted into a new microfuge tube and 0.1 volume of NaOH (3M) was added, mixed and then 0.33 volume HCl (1M) was added before using immediately.

2.2.2.4 Hybridisation of labelled probe to DNA on membrane

To prepare the membrane for hybridisation it was soaked in prehybridisation solution (500µl 100 x Denhardt, 500µl 10% SDS, 2.5ml 20 x SSPE (6M NaCl, 01M sodium

phosphate, 0.02M EDTA pH7.7), 6.5ml dH_20 and 40µl 5mg/ml salmon sperm, denatured for 5 min at 94°C) rotating in a glass drum for 1h at 65°C in a Techne oven. The prepared probe (section 2.2.2.2) was added to the prehybridisation buffer through a port hole in the drum. This was left to hybridise over night. In the morning the hybridisation buffer was discarded and the membranes were washed at 65°C in 0.2% SSPE 0.1%SDS for 2 x 10 min. They were then washed in 65°C 0.2% SSPE 0.1% SDS at 65°C for 2 x 10 min. The membranes were wrapped in cling film and placed DNA side up in a film cassette lined with intensifying screens for detection.

2.2.2.5 Autoradiography

Fuji X-ray film was placed on the membrane in the cassette and stored at –70°C .

Depending on the intensity of the signal, the film was developed after 1-3 days in a Fuji X-ray film processor.

2.2.3 Cloning.

2.2.3.1 Purification

The 425bp PCR product obtained from the nested *Xenopus* primers on the *A.quercuscalicis* template were chosen to be cloned. Several PCR reactions set up using the optimised programme and the products were then eluted from the 1.7% TBE agarose gel (section 2.2.1.2). These were purified using a PCR purification kit. This involved placing the eluted band into a Wizard minicolumn and centrifuging for 5 min. The supernatant was precipitated and resuspended in a smaller volume. This was achieved by ethanol precipitation which required adding 0.1 volumes of NaAc pH 4.8 and 2.5 volumes of ethanol to the product of the spinning purification. This was left in a -20°C freezer for

30 min and then spun for 30 min in a 4°C microcentrifuge. The supernatant was removed, 1ml of 70% ethanol added and spun for 10 min. The supernatant was removed and the pellet was resuspended in 20µl of TE (10mM Tris HCl pH7.6, 1mM EDTA pH8.0).

2.2.3.2 Ligation

The purified product was ligated with the pGEM™-T Easy vector from Promega, shown in Figure 2.3). The following ligations were set up: 1) A standard control, 1µl T4 DNA ligase buffer, 1µl pGEM™-T Easy, 7µl (7ng) PCR product, 1µl T4 DNA ligase (3units/µl). 2) positive control, 1µl T4 DNA ligase buffer, 1µl pGEM™-T Easy, 2ul control insert DNA, 1µl T4 DNA ligase (3units/µl) and up to 10µl with dH_2O). 3) Background control, 1µl T4 DNA ligase buffer, 1µl pGEM™-T Easy, 1µl T4 DNA ligase (3u/µl) and up to 10µl with dH_2O. The ligations were left overnight at 14°C.

2.2.3.3 Transformation

The ligations were used to transform *E.coli* cells by electroporation. The following were set up: 1) 2µl of the standard control and 50µl of competent cells 2) 2µl of the standard control and 50µl of competent cells, 3) 2µl of the standard control and 50µl of competent cells, 4) 2µl of the standard control and 50µl of competent cells, 5) 2µl positive control and 50µl of compotent cells 6) 2µl positive control and 50µl of compotent cells 7) 2µl background control and 50µl compotent cells 8) 2µl background control and 50µl compotent cells 9) 0.1ng uncut plasmid and 50µl of competent cells 10) 0.1ng uncut plasmid and 50µl of competent cells .These were transferred to separate cuvettes and electroporated at 2.5 volts with a resistance of 200 ohms and capacitance of 25, 800µl of

LB was immediately added. These were left at 37°C for 45 min and then spun down at 1000g for 10 min and resuspended in 200µl LB. 100µl x 2 of each transformation was plated on LB-ampicilin plates with IPTG and X-gal for blue/white screening. These were left overnight at 37°C. White colonies from the standard control were streaked out for single colonies on LB-amp plates and then single colonies from these plates were grown overnight in 2ml LB-amp liquid cultures.

2.2.3.4 DNA extraction of the transformed colony

The over night cultures were spun down and resuspended in 50µl of sterile water, 350µl STET (100mM NaCl, 10mM Tris pH 8, 1mM EDTA pH 8, 5% Triton X-100) and 25µl of 10mg/ml lysozyme. This was mixed and placed in boiling water for 1 min. This was spun for 30 min and the pellet was removed and 30µl of 3M NaAc was added. After mixing 350µl of isopropyl alcohol and left at room temperature for 15 min. After spinning for 10 min the resulting pellet was washed with isopropyl alcohol and resuspended in 50µl of TE.

2.2.3.5 Digestion of plasmid DNA

EcoR1 digests were set up for all the plasmid DNA preps using 1µg DNA, 1µl of EcoR I enzyme, 2µl of the appropriate enzyme buffer and made up to 20µl with dH_2O. This would cut out any insert that may be present and the true positive clones could be identified. These were run on 1.7% TBE agarose gels (see section 2.2.1.2)

2.2.3.6 Sequencing

The positive clone was sent away for sequencing to The Advanced Biotechnology Centre,

The Charing Cross and Westminster Medical School. The sequence was analysed using BLAST (Altschul *et al.*, 1990).

2.3 Analysis of Proteins

2.3.1 Extraction of protein

Inner-gall and callus tissue were used for protein extraction. The inner-gall tissue was dissected from the gall and immediately frozen in liquid nitrogen or dry ice and stored at –70°C or used immediately for extraction. The callus was used for extraction immediately following the bioassay. The relevant tissue was ground in a mortar and pestle with dry ice and then placed in a microfuge tube and centrifuged with lid open for approximately 1min to remove any remaining dry ice. 20-30µl of the extraction buffer (18.8ml 0.5M tris pH 6.8, 6g SDS, 15ml glycerol, 7.5ml ß-2-mercaptoethanol, 15mg bromophenol blue, up to 50ml with dH_20) was added and vortexed well. The samples were then boiled for 15 min and centrifuged at 13,000rpm for 20 min at 4°C to pellet the debris. The supernatant was transferred to a clean tube and stored at –20°C until required.

2.3.2 Determination of protein concentration

To determine concentration of protein from the extractions a BioRad protein assay dye reagent was used. This is based on the colourimetric method described by Bradford (1976). 2µl of the sample were mixed with 5ml of 1x reagent in a plastic cuvette and left at room temperature for 5 min before taking the absorbance at 595nm. Known concentrations of Bovine serum albumin (BSA) were mixed with the reagent and absorbance readings taken to give a standard curve. The sample concentration could be estimated by interpolation from the standard curve.

2.3.3 SDS-PAGE of proteins

SDS-PAGE was used to analyse the protein extracts made from the callus and inner-gall tissue. This was carried out using gradient gels run on BioRad mini gel electrophoresis equipment, which enables 2 gels to be run simultaneously using the discontinuous buffer system (Laemmli 1970). Gradient polyacrylamide gels were prepared from stock solutions of 20% acrylamide (19.65ml of 32:1 acrylamide:bis (N,N'-mebthylene bisacrylamide), 17.85ml 1M Tris pH8.8, 600µl 10% SDS , 11.9ml 50% glycerol) and 6.4% acrylamide (7.15ml of 32:1 acrylamide:bis, 17.85ml 1M Tris pH8.8, 600µl 10% SDS , 24.4ml dH_2O) The gels were poured using a grid intermixing chamber that evenly mixed the two stock solutions giving an even increase in gradient. In each chamber of the gel pourer 1µl TEMED (N,N,N,N'-tetramethyl ethylene diamine) and 20µl 10% ammonium persulphate was added to each stock solution in order to catalyse polymerisation once poured. The gels were left to set for 45 min at room temperature with water saturated butanol on top to ensure a flat surface. The stacker gel (5ml of 32:1 acrylamide:bis, 7ml of 1M tris pH6.8, 500µl of 10%SDS, 38ml dH_20, 5µl TMED and 150µl APS) was poured on the gel using a plastic comb to create wells. The protein samples to be loaded were mixed with 5µl 2x SDS sample buffer (18.8ml 0.5M tris pH 6.8, 6g SDS, 15ml glycerol, 7.5ml ß-2-mercaptoethanol, 15mg bromophenol blue, up to 50ml with dH_20) and then denatured at 94°C for 5 min before immediately loading onto the gel. A wide range molecular weight marker from Sigma was always loaded next to the samples to indicate the size of the proteins being analysed. The gel was run in a gel tank with a discontinuous buffer system containing electrode buffer (3.03g Tris, 14.42g glycine, 1g SDS, up to 1L with dH_20) and run between 80-150V until the dye front had

run off the gel.

2.3.3.1 Staining SDS-PAGE gels with Coomassie Brilliant Blue

The gel was then removed from the apparatus once it had finished running and stained with Coomassie Blue by submerging in staining solution (40% methanol, 10% acetic acid, 0.1% Coomassie Blue) for 4-12 h on a rotating platform. The gel was then removed and placed in destain (40% methanol 10%acetic acid 50% dH_20) for 4-12 h. The destained gels can be visualised on a light box.

2.3.4 Analysis of Protein

2.3.4.1 Western blot transfer

The protein samples to be tested using hybridisation were run on a gradient SDS-PAGE gel (section 2.3.3). To blot the proteins onto the membrane a semi-dry electro blotter was used. For each gel, 2 pieces of thick filter paper and a piece of Hybond C membrane was cut to fit the gel. The filter paper and membrane were soaked in western transfer buffer (5.28g trizma base, 2.93g glycine, 3.75ml 10%SDS, 200ml methanol, up to 1L with dH_2O) and a the blot was constructed on the semi-dry blotter by placing a piece of filter paper on the base, followed by the Hybond C membrane, the SDS-PAGE gel and finally the second piece of filter papers. The air bubbles were removed and excess transfer buffer wiped dry before placing the top electrode and safety cover of the blotter. This was run at a constant amperage of 0.25 A for 15 min for 1 gel or 25 min for 2 gels. After the transfer, the blot was dismantled and the membrane immediately blocked overnight at 4°C in blocking solution (PBS, 0.1% Tween-20, 5% dried skimmed milk).

2.3.4.2 Analysis of proteins bound to membrane

To detect biotinylated proteins, streptavidin-horseradish peroxidase was used, this hybridises to the biotin on the protein which can then be detected using ECL (section 2.3.4.3). The membranes were washed for 3 x 5 min in PBST and streptavidin-horseradish peroxidase was added to the final wash (1:10000 dilution) and incubated for 30 min at room temperature gently shaking. The membrane was washed for 3 x 10 min in PBST and detection was carried out using ECL.

To detect protein disulpphide isomerase (PDI), formate dehydrogenase (FDH) the membranes were washed for 3 x 5 min in PBST and incubated with the primary antibody anti-PDI and anti-FDH (raised in rabbits) at a 1:1000 dilution for 1 h. The membrane was washed for 3 x 5 min in PBST and incubated with goat anti-rabbit horseradish peroxidase secondary antibody at a 1:100000 for 1 h. The membrane was washed for 3 x 10 min in PBST and detection was carried out using ECL.

To detect the AGPs using JIM4, JIM13 MAC207 and LM4 a method adapted from that used by Smallwood *et al.* (1994) was followed. The gall was stamped onto the Hybond-C membrane and incubated for 1 h in blocking solution. This was washed for 3 x 5 min in PBST and incubated with the respective primary antibody (raised in rat) at a 1:1000 dilution for 1 h. The membrane was washed for 3 x 5 min in PBST and incubated with goat anti-rat horseradish peroxidase secondary antibody at a dilution of 1:100000 for 1 h. The membrane was washed for 3 x 10 min in PBST and detection was carried out using ECL.

2.3.4.3 ECL detection

An ECL detection kit (Amersham Pharmacia Biotech) is an enhanced chemiluminescence method achieved by the oxidation of luminol by the horseradish peroxidase attached to the streptavidin or secondary antibody. Where the antibody has bound to the membrane, the ECL chemicals will oxidise and then emit light. The light emmited from the luminol can be detected by exposing it to X-ray film to determine where on the membrane the antibody has bound.

The ECL procedure entailed mixing equal volumes of reagent 1 and reagent 2 together to give and final volume of 0.125ml/cm^2 membrane. The reagents were poured on the membrane and left for exactly 1 min before draining the membrane and wrapping in cling film. The membrane was exposed to X-ray film for 10 sec, 15 sec, 30 sec, 1 min and 2 min (and longer if necessary) and developed in a Fuji X-ray film processor.

2. 4 Cell Biology techniques

2.4.1 Tissue sectioning

2.4.1.1 Fixing

For sectioning the tissue was fixed in buffered formaldehyde. This entailed firstly taking freshly collected galls and dissecting out their larval chambers. In the galls where the chambers can easily be separated from the outer gall, the chambers were removed and either cut in half and the larva removed, or kept whole and had the sclerenchyma sheath removed from the outside. For multilocular galls, and galls where the chamber could not easily be separated from the outer gall, a small section of tissue was taken containing the

chamber. The larval chambers were placed in fixative solution for 3 h at room temperature. The fixative solution was made from 3ml freshly prepared formaldehyde (4g paraformaldehyde in 60ml dH_20 heated to 65°C and stirred until dissolved) 12.5ml of 0.1ml phosphate buffer pH7 (0.1M sodium phosphate pH7.4, containing 0.9% (w/v) sodium chloride) and 6.5ml ddH_20. The solution was altered to pH7. The fixed material was washed in PBS at room temperature for 3 x 5min and then placed in cryoprotection solution (10% (w/v) sucrose in PBS, 20µg/ml sodium azide).

2.4.1.2 Slide preparation

Slides were coated in Vectabond which enables the section to stick to the microscope slide. The slides were first washed in acetone for 5 min, then placed in Vectabond (7ml Vectabond 343ml acetone) for 5 min and then washed in dH_20 for 30 sec before leaving to dry.

2.4.1.3 Sectioning

Fixed larval chambers were sectioned using a Leica cryostat model CM1800, which had an operating temperature of -15°C and a cutting angle of 2.5. The fixed material was embedded in Tissue-Tek (O.C.T. 4583) and frozen in liquid nitrogen. The Tissue-tek block was then mounted onto a disc with some more Tissue-Tek and placed into the cryostat. The block was orientated and then sections between 10µm-25µm thick were taken. The sections were collected onto the Vectabond coated microscope slides and left to air dry for 1h at room temperature before placing at –20°C in a sealed container with silica gel as a desiccant. To analyse the sections, a drop of mounting medium containing DAPI was placed on the slide, a coverslip applied and the slides were visualised using a

Leitz Aristoplan fluorescent microscope.

2.4.2 Immunohistochemistry

To detect the biotinylated protein, the tissue section slides were first washed in 0.1M phosphate buffer pH 7 for 3 x 5min to remove Tissue-Tek, incubated slides in 0.1M phophate buffer with 1% blocking agent (milk powder) before incubating with Cy3-conjugated streptavidin peroxidase for 1 h at 20°C. The slides were washed for 3 x 5 min in 0.1M phosphate buffer pH 7 and then mounted using antifade mounting medium containing 10% DAPI, before examined using a Leitz Aristoplan fluorescent microscope.

2.4.3 Protoplast Preparation

Inner-gall tissue was dissected from *B.pallida* and fixed in 3:1 ethanol: acetic acid for 5 h at room temperature and then placed at –20°C until required. The protoplast preparation of the fixed material was carried out as follows: the fixed material was placed in a microfuge tube and 1ml of 1 x enzyme buffer (4mM citric acid, 6mM sodium citrate in dH_20) was added to the tube. This was spun down briefly and the buffer removed. This wash with enzyme buffer was repeated 3-5 times to remove all the fixative. The material was then placed in a digestive enzyme and left in a 37°C water bath for 40 min. The material was washed with enzyme buffer at least 5 times by removing 500µl of enzyme and adding 500µl of enzyme buffer, spinning for 5 min at 80g and then removing 500µl again. This was repeated several times to remove all the enzyme. The material was fixed by removing 100µl of the buffer and adding 100µl of freshly prepared 3:1 ethanol:acetic acid. This was spun for 5 min at 80g and then 200µl of buffer was removed and 200µl of fixative was added. This was repeated a number of times until the material was in total fixative. A

small drop of these freshly prepared protoplasts was dropped on to a chromic acid washed microscope slide and a drop of acetic acid was added. A coverslip was placed over the material and then using my thumb I firmly pressed down to squash the nuclei. The preparations were observed using a light microscope to find a good slide showing intact nuclei. Chosen slides were placed on dry ice for 10 min so the cover slip could be flicked off and then left in the oven at 37°C over night.

2.4.4 Fluorescent In-Situ hybridisation (FISH)

2.4.4.1 Labelling of probe

To label the 18s, 26s, 5.8s rDNA for use in FISH, nick translation labelling was used. 1µg of 18s, 26s, 5.8s rDNA in 33µl was mixed with 5µl 10 x nick translation buffer, 5µl unlabelled dCTP, dATP, dGTP mix (0.5mM stocks of each mixed 1:1:1) , 1µl Dig-11 dUTP-dTTP (1mM dig-dUTP and 1mM dTTP mixed 2:1), 1µl of 100mM DTT (dithiothreitol). This was mixed and briefly spun down before adding 5µl DNA polymerase/DNase mix. This was mixed and spun down and incubated for 1.5 h at 15°C. This was then ethanol precipitated by adding 2.5 volumes of absolute ethanol and 0.1 volume of NaAc pH 4.8 and placed at –20°C over night. This was spun down at 4°C for 30 min and the supernatant removed. The pellet was washed in 70% ethanol several times before air drying and resuspending in 10µl TE.

To check the digoxigenin label had incorporated into the DNA a dot blot was carried out using a DNA detection kit from Life Technologies which contained 3 main buffers:

Buffer 1	0.1M Tris HCl pH 7.5 0.15M NaCl
Buffer 2	0.5% (w/v) blocking reagent (Boehringer Manheim) in buffer 1. Dissolved by heating to 55°C for 1h.
Buffer 3	0.1M Tris HCl pH 9.5 0.1M NaCl 0.1M $MgCl_2$

A small piece of Hybond N membrane was soaked in buffer 1 for 5 min and blot dry between filter paper. .1µl of DNA probe was loaded onto the membrane and left to dry for 10 min. The membrane was placed in buffer 1 for 1 min and then in buffer 2 for 30 min, shaking gently. The membrane was drained and 5ml of anti-digoxygenin antibody solution (diluted 1:5000 in buffer 1) was poured over the membrane and incubated at 37°C for 30 min. The membrane wash then washed in buffer 1 for 3 x 5 min and transferred to buffer 2 for 2 min. 5 ml of NBT/BCIP detection reagent (22.5µl NBT, 17.5µl BCIP in 4.96µl buffer 3) was poured on the membrane and left for 5-10 min in the dark for colour to develop. The membrane was then washed in water and left to dry. If the probe had successfully labelled then a black dot was visible on the membrane.

2.4.4.2 Pre-treatment

In Situ was carried out as follows: 100µl of 0.2mg/ml of RNase A was added to each slide and a plastic cover slip applied and incubated for 1h at 37^0C. The slides were washed in 2

x SSC (0.3M NaCl and 30mM sodium citrate) for 3 x 5 min and then incubated in 0.01M HCl for 3 min. They were then incubated in 100µl of 1µg/ml pepsin for 5 min at 37^{0}C and washed in H_20 for 2 min then in 2 x SSC for 3 x 5 min. The slides were fixed in formaldehyde freshly prepared from paraformaldehyde (2g in 40ml water in 60°C bath for 15 min and clear with 10ml of 0.1M NaOH) at room temperature for 10 min and washed in 2 x SSC for 3 x 5 min. They were dehydrated through 70%, 90% and 100% alcohol for 3 min each and the slides were then air-dried.

2.4.4.3 Denaturation

Denaturation was carried out as follows: Probes were heated to 75°C for 15 min, vortexed, pulsed and placed on ice immediately. Probe composition: 20µl 100% formamide, 8µl 50% dextran sulphate, 4µl 20 x SSC, 0.5µl SDS, 0.5-1µl probe-Dig, 0.5-1µl probe-Biotin, up to 40µl total volume with dH_20.

Slides were denatured in 70% formamide (15ml 2 x SSC and 35ml formamide) at 68^{0}C for exactly 2 min and then immersed in 70% ice cold alcohol for 5 min, then 90% and 100% and left to air dry.

2.4.4.4 Hybridisation

Hybridisation was achieved by adding 40µl of the digoxegenin labelled 18s-5.8s-26s (pTa71) probe to each slide and incubated overnight at 37°C in a humid chamber. The slides were then washed in 2 x SSC for 3 x 5 min at 37°C.

2.4.4.5 Washing

Washing of the slides was carried out in 20% formamide in 0.1 x SSC at 42°C for 2 x 5 min. The slides were then washed in 2 x SSC for 3 x 3 min at 42°C. The slides were then washed in 4 x SSC Tween (0.2% Tween) for 5 min at room temperature.

2.4.4.6 Detection

Detection was achieved by first draining the slides and blocking with 100µl of BSA at room temperature for 5 min and then applying a plastic cover slip. The block was drained and 50µl of digoxygenin detection reagent (3µl of 200µg/ml stock FITC-antidig in 297µl BSA) was applied and incubated for 1h at 37°C in the humid chamber. The slides were washed 4 x SSC Tween for 3 x 5 min and drained before 100µl (2µg/ml) of counter stain DAPI (10%) was applied to each slide for 10 min and were briefly washed in 4 x SSC Tween. A drop of antifade was added and a glass cover slip applied. To observe the probes a Leitz Aristoplan fluorescent microscope was used.

2.5 The bioassay

2.5.1 Callus formation

To obtain callus from *Rosa rugosa,* leaves were left overnight under running water and then sterilised in a laminar flow hood: 30 sec in ethanol, 20 min in 20% bleach, 3 x 5 min in ddH_20. The leaves were then injured using a sterile scalpel and cultured on sterilised Murashige and Skoog (MS) solid media. The cultures were left at 24^{0}C for approximately 4 weeks. The callus which formed was removed and placed in liquid MS media. The volume of cultures were either 50ml of media in a 100ml flask or 200ml of media in a 500ml flask. The cultures were sealed with sterile cotton wool, sterile aluminium foil and

masking tape. They were kept in the dark at 24°C on a horizontal shaker and subcultured every 1-2 weeks.

Oak leaves and shoots of *Quercus robur* were also used with a more vigorous sterilisation technique. In addition to the standard sterilisation technique anti bacterial and anti fungal agents were used. Nystatin and spectinomycin were first used in 2 of the final washes at 5mg/500ml and 10mg/500ml respectively. This was not successful so the concentration of nystatin was increased to 25mg/500ml and gentamycin was used at 25mg/500ml. An alternative method was used which entailed standing shoots with leaves in a solution of nyastatin (25mg/500ml of 0.1M K_2HPO_4) for 2.5 days before carrying out the standard sterilisation technique.

2.5.2 Gall Collections

Samples of *Diplolepus spinosa* larva were kindly collected and sent from Canada by Joe Shorthouse. And these were used for the initial bioassays. The additional collections of *B.pallida* were made at Putenham Common and Barn Hill in Wembly over a period of 5 weeks in spring to ensure galls of all developmental stages. In August *A. quercuscalicis* were available for collection and several collections were made over a period of 6 weeks and the samples snap frozen in liquid nitrogen and stored at –70°C until needed.

2.5.3 Larval extracts

Extracts from *D.spinosa* were made using 70mg of larvae, grinding these in dry ice and adding 500µl of dH_20. This was vortexed well and then kept at room temperature for 20 min, vortexing at intervals. The mixture was centrifuged for 30 min at 13,000rpm at 4°C

and the supernatant was removed and used as the larval extract.

Larvae from *B.pallida* and *A.quercuscalicis* gall first needed to be dissected from the galls. This entailed cutting or tearing open the galls and then for *B.pallida* removing the large number of larvae under a dissecting microscope. The larvae were immediately frozen in a microfuge tube in dry ice and stored at –70°C until required. *A.quercuscalicis* larvae were slightly more difficult to dissect as they only contain one larva per gall and the chamber is often protected by a sclerenchyma layer, making it difficult to open gently. These larvae were frozen on dry ice and stored at 70°C until required. Extracts from *B.pallida* and *A.quercuscalicis* larvae were obtained from 200mg of larvae following the procedure described above.

2.5.4 Standard bioassay procedure

For a standard bioassay, 100mg of callus were placed in a sterile glass vial with 500µl of MS media containing all the growth regulators as stated above. The extract being tested was added to the glass vial using a syringe through the resealable lid. The amount of extract used varied according to the assay. The vials were stored at 24°C on an orbital shaker for 16-18 h. The callus was then removed from the vial and used for protein extraction.

2.5.5 Size fractionation of larval extract

The extract was placed through a 0.2µm syringe filter that had been pre wetted using dH_20. The filtered and the non-filtered were used in the standard bioassay.

Further size fractionation was achieved using centricon spin columns (Amicon) with 30kDa and 100kDa cut off points. The spin columns were first washed by centrifuging in a Beckman J2-21 in rotor JA 20 with 500µl of dH_20 for 5 min at 6,000rpm, inverting the tube and centrifuging for 2 min at 2,500rpm to collect the retentate that always remains above the membrane. This wash was repeated to ensure that all the glycerol was removed form the membrane. Then 1ml of larval extract was placed in the spin column and centrifuged for 30 min, the column was then inverted and the retentate was collected in the cap by spinning for 2 min at 2500rpm. The sample which passed through the column membrane in the first spin was used as the larval extract less than 30kDa or 100kDa in the bioassay, and the retentate was used as the sample larger than 30kDa or 100kDa in the bioassay. These fractions were applied to the standard bioassay.

2.5.6 Fraction of larval extract with Con A

Concanavalin A (Con A) binds reversibly to glycoproteins, polysaccharides and glycolipids to enable their separation and purification from solutions. To prepare the Con A Sepharose Lab Pack (Pharmacia Biotech), I degassed 1ml of sepharose using a vacuum pump. The sepharose was then poured down a glass rod into the Con A column to prevent any air pockets. The column was immediately filled with Con A buffer and this was left to flow through. Before the buffer had completely flowed through the larval extract sample was added. This was then washed through will 3 x 1ml of Con A buffer, collecting all of the flow through in mirofuge tubes. Elution buffers of varying molarity (0.1M, 0.15M, 0.2M, 0.4M) of a-d-methylmannoside were prepared in Con A buffer and passed through the column and the flow through collected separately. The collected samples were used for a standard bioassay.

2.5.7 Fractionation of larval extract by HPLC

An anion exchange Mono Q column (Pharmacia Fine Chemicals) was used for the fractionation of the larval extract. The column was equilibrated with 20mM tris pH 7.5 that had been placed through a 0.2µm filter. The other tris buffer 500mM NaCl in 20mM tris pH 7.5 was also filter sterilised. Larval extract (240mg/100µl) was filtered through a 0.2µm filter and then injected into the column. Bound proteins were eluted using an increasing gradient of NaCl up to 500mM. The samples were collected according to peaks observed on a trace, which was showing the absorbance at 280nm. A total of 35 fractions were collected in volumes ranging from 200µl-1.7ml. To test them I had to concentrate them and remove any trace of salt from the buffer. To achieve this I used half of each fraction and spun the fraction through a 10kDa microcon spin column. This entailed washing the column with dH_2O for 2 x 5 min at 13,000rpm in a microcentrifuge. The fractions were then placed in the spin column, the maximum volume for the column was 500µl therefore fractions with volumes greater than 500µl were spun twice using the same column. The fractions were spun for 30 min at 13,000rpm and the flow through was thrown away. The filters were inverted in a new microfuge tube and spun for 3 min at 3,000rpm. Some filters did not have a retentate, therefore I added 10µl to the filter and agitated for 30 sec and then sucked off using a Gilson p20. The concentrated fractions were made up to 100µl with dH_20 and used in the standard bioassay.

The anion exchange fractionation was repeated using an increase in the molarity of NaCl and less fractions. The column was prepared as before. This time the column was pumped with 1M NaCl to remove any bound molecules on the column and then equilibrated using

the 20mM tris pH7.5. 150µl of larval extract (200mg/150µl) was injected into the column and collections were made every 2 min. A steeper gradient of NaCl was used going up to 1M NaCl. The immediate flow through was collected and a total of 13 fractions were collected. The fractions were concentrated using Amicon spin columns with 30kDa cut off (as described above). The concentrated fractions were made up to 100µl with dH_2O and used in the standard bioassay.

2.5.8 Fractionation of larval extract by separating head and body

200mg of *A.quercuscalicis* larvae were dissected and then used to separate the head and body of larvae. This was achieved by dissecting in a petri dish placed on a thin block of dry ice, to keep the larvae frozen, and using a frozen scalpel blade. The heads were removed and immediately frozen onto the scalpel blade from where they could be placed in a microfuge tube in dry ice. The bodies were placed in a separate tube on dry ice. Extractions were made from each the head and body using the standard method in 500µl of dH_20. Unfiltered extracts from the head and body were used for the standard bioassay.

2.5.9 Controls

2.5.9.1 Biotinylation

To biotinylate the larval extract an Amersham biotinylation module was used. 100µl of biotinylation reagent was added to 2.5mg (1mg/ml) of extract and incubated at room temperature while shaking. The Sephadex G25 column was equilibrated using 5ml PBS pH7.5 containing 1%BSA, followed by 20ml PBS pH7.5. The larval extract was added to the column and eluted using 5ml of PBS pH7.5. Fractions were collected and UV absorbance at 280nm was used to identify the fractions containing the biotinylated larval

extract. These were used in the biotinylated bioassay.

2.5.9.2 Non-gall forming herbivorous larvae

Larval extracts using 200mg of *Profonuca pygmea* larvae (leaf miner) in 500®µl of dH_2O were prepared using the same larval extract method and 100µl was used in the standard bioassay.

2.5.9.3 Jasmonic acid

JA was also applied to callus as in the standard bioassay using a range of concentrations from 0.1nM-2µM. The bioassay was left for 24 h and 48 h at 24^0C on an orbital shaker. Protein analysis was carried out as usual.

3: Do Cynipid gall wasps use Nod factors to reprogramme plant development ?

Introduction

3.1 Nod factor synthesis and role in plant signalling

The successful and long-standing symbiotic relationship between rhizobia and legumes demonstrates the ability non-plant organisms have to communicate with, and override the developmental pathway in plants. Rhizobia use specific signalling molecules to achieve what has been described as a "reciprocal molecular conversation" with the host plant, resulting in the nodulation of the root, and is reviewed below (Fisher and Long, 1992). In cynipids, the molecular conversation is still to be translated; however, wasps are in some way communicating with the plant, which responds and obeys instructions given by the insect. It is not known if the same signals are being used in the cynipid molecular conversation as those with rhizobia, although it is possible that the two non-plant organisms use identical, or at least similar molecules, to achieve communication.

Unlike gall formation, all aspects of the rhizobia-legume interaction have been analysed and the signalling molecules produced by the rhizobia to initiate and control nodulation in the host are lipo-chitooligosaccharides known as Nod factors. Nod factors induce root hair deformation, cell division, infection thread formation and gene expression in the host legume root, but are also believed to have general signalling properties, as discussed in chapter 1. The possible widespread signalling properties of Nod factors lead us to question if cynipids produce Nod factors to alter the development of the host during gall formation.

Another parasite-plant interaction which has also been compared to nodulation, and for which the use of Nod factors has been speculated, are the root knot and cyst nematode (Bird, 1996). The nematodes induce galls and syncitia respectively, on the root of the host to house and feed the developing larva until adulthood. The eggs are laid in the soil by the female adult and the second stage juveniles infect the root and migrate to the vascular bundles where the induction of the feeding cells from the xylem cells occurs. The sedentary larvae go through three moults and emerge as adults to begin the cycle again. There is a constant stimulus from the larva throughout development as, if the larva is killed, the feeding cells die (Bird, 1962). The specialised feeding cells, known as giant cells and syncitia in the root knot and cyst nematode respectively, surround the larva providing nutrients. They are multinucleate and are formed by multiple rounds of DNA synthesis without cytokinesis in the root knot gall and by cell dissolution in the cyst gall (Engler *et al.*, 1999). The cells adjacent to the feeding cells multiply and form the root gall. These outer cells are not multinucleate and do not show the distinct characteristics of the feeding cells. The giant cells or syncitia, as well as being multinucleate, have cell wall ingrowths, are cytoplasmically dense, have fragmented vacuoles and high metabolic activity (Jones and Northcote, 1972).

Despite the extensive research carried out on nematodes, the details of the whole process remain unclear. It is known that a secretion from the stylet of the nematode is responsible for initiation, although the signals that induce and control the process have not been determined (Jones and Northcote, 1972). A gene expressed in rhizobial root nodules, ENOD 40, is also expressed in the nematode gall cells, which lead Bird (1996) to question

if active Nod factors may also be produced by the nematode to induce gall and syncitia. There is no direct evidence that they produce Nod factors, although the nematode egg contains chitin, demonstrating that is has the necessary genes to form chitin oligomers (Bird, 1996). Also, a nodL homologue, an O-acytyltransferase, involved in the production of Nod factors, has also been found in the nematode genome (Bird and Koltai, 2000). If Nod factors are involved, the signalling pathways appear to have diverged, as demonstated in the nematode host plant *Melilotus alba.* When this is mutant in the genes necessary for nodulation, giant cells can still form when infected with the nematode *M.incognita* (Bird, 1996).

As the nematode, rhizobial and cynipid host interactions all represent examples of reprogramming of host development to protect and provide nutrients for the invading organism, although each achieves this in specific ways, it is possible a common signal is involved. This possibility lead me to search for a nod gene homologue within the gall wasp genome using PCR. A homologue within the genome would indicate if the wasp is capable of producing the chitooligosaccharide signal which could then be investigated as a signal during gall formation. *NodC* was chosen as the sequence to search for as it is common to all rhizobia and homologues to *nodC* have been discovered in many organisms. Their products are believed to have an important signalling role in development. To understand the importance of Nod factors and their signalling properties in detail, this will be discussed together with a description of nodule formation before explaining the search for *nod*C in the cynipid gall wasp genome.

3.1.1 Nodulation

Nodulation is a complex process which involves several steps before the final nitrogen-fixing nodule appears. The signalling molecules involved are flavonoids, secreted by the host, and Nod factors, chitin oligomers secreted by the rhizobia. The whole process is initiated by the host plant emitting flavonoids into the soil where surrounding rhizobia are able to detect specific flavonoids and, as a chemoattractant, they draw the compatible rhizobia towards the roots. *Nod* gene expression is activated by the flavonoids and the rhizobia start to produce Nod factors and these have a positive feed back response on flavonoid production, ensuring the attraction of more rhizobia (DÈnarie and Debelle, 1996).

The rhizobia attach themselves to the tips of certain root hairs, depending on their stage of maturity and their position. It is believed that only root hairs within zone II of the root are those able to react to Nod factors. Zone II root hairs are young but have almost reached full maturity, lack a clear zone and large organelles, which are usually found in zone I root hairs (Heidstra *et al*,, 1997). When Nod factors come in contact with these zone II root hairs then reinitiation of tip growth occurs and again the clear zone can be observed and cytosolic Ca^{+} concentration gradient is built up as seen in the tip growing hairs of zone I (Heidstra *et al*., 1997). The new tip growth is part of the root hair deformation, where the root hair tip curls around encapsulating the rhizobia aiding the infection process which soon follows. Nod factors alone are able to initiate the deformation process but rhizobia are needed for curling (Heidstra *et al*., 1997).

The actual infection of the root is achieved via the infection thread and preinfection

thread, formed from the hydrolysis of plant cell walls and invagination of the plasma membrane followed by deposits of new cell wall (Brewin, 1991). The rhizobia are able to infect the root through these tubular structures that go through the root hair cell, into the cortex and into the nodule primordia. The formation of these infection threads is initiated by Nod factors and is achieved by the reactivation of cortical cells and initiation of certain host early nodulin genes (ENOD genes) for the production of proteins used to form the infection thread. Simultaneous to infection thread formation, nodule primordia are initiated as the cortical cells resume division.

The position of the nodule primordia are normally in the cortex and these eventually form the functioning nodule (Heidstra *et al.*,1997). The concentration of Nod factors needed to induce primordia formation and gene expression is 10^{-7} M which is greater than the concentration needed to induce root hair deformation, 10^{-9} -10^{-12} M (Heidstra *et al.*, 1997).

3.1.2 Nod genes and Nod factors

The rhizobial response begins with the flavonoids binding to NodD, a constitutively expressed rhizobial DNA binding protein, which regulates the expression of other inducible *nod* genes involved in nodulation. NodD interacts with regulatory regions, known as nod boxes, found upstream from the inducible *nod* genes (Rostas *et al.*, 1986). It is believed that the Nod D protein, when bound to flavonoids, binds to the nod boxes and causes the DNA to bend allowing access by RNA polymerase, and so the induction of expression of *nod* genes (Fisher and Long, 1993).

There are a total of 50 *nod* genes involved in the entire nodulation process, 16 of which are involved in Nod factor biosynthesis and transport (Freiberg *et al.*, 1997). The common nod genes, those found in all rhizobia, are *nodABC I* and *nodJ*. *nodI* and *nodJ* gene products are believed to belong to the family of ATP-binding cassette (ABC) transporters, membrane pumps responsible for the secretion of Nod factors (Mergaert *et al.*, 1997). *Nod* ABC are responsible for the production of the basic Nod factor, a _ 1-4 N-acetylglucosamine oligomer of 4-6 residues. NodC is a chitin oligomer synthase and is responsible for producing the chitooligosaccharide from substrates such as UDP-N-acetylglucosamine, acetyl-CoA, S-adenosyl-L-methionine or acyl-ACP (Mergaert *et al.*, 1997). NodB deacetylates the non-reducing end of the terminal residue (John *et al.*, 1993) and NodA then transfers a fatty acid to this position (Heidstra and Bisseling, 1996).

The resulting Nod factor is further modified according to the *nod* genotype of the rhizobia and these specific alterations are vital for regulating the range of host plants the rhizobia can infect. The main differences between the Nod factors responsible for affecting their host range are the number of residues linked to form the chitin oligomer backbone, the number and position of double bonds in the fatty acid and the additional modifications made to the reducing and non-reducing end (Mergaert *et al.*, 1997). Many of the other *nod* genes are responsible for making these additional modifications to the backbone, which may be vital for host specificity. For example, NodX is responsible for the addition of an O-acetyl group at the reducing end and others such as NodH, NodP and NodQ are important for regulating the host specificity. By using strains which are mutants for these genes it has been shown that host specificity range can be altered by the removal of only one *nod* gene. For example, NodH is believed to have sulphotransferase ability (Schulze *et*

al., 1995) and mutants for this gene in *Rhizobium meliloti* lose the ability to infect their original host alfalfa, however, gain the ability to nodulate a normally non-host legume vetch (Heidstra and Bisseling 1996). Mutants of NodPQ do not lose their ability to nodulate their regular host but, again can nodulate non-host species (Cervantes *et al.*, 1986; Faucher *et al.,* 1989).

It has been suggested that a correlation may exist between the type of nod factor produced and the type of nodule formed (Spaink, 1996). The Nod factors can be grouped according to their structure, tetrameres and pentamers with specific fatty acids with certain double bonds are formed by bacteria such as *Rhizobium meliloti* and *R.leguminosarum.* Another kind are pentamers which have an N-methyl group on the N atom carrying the fatty acid (Mergaert *et al.,* 1997). These two groups of Nod factors may correlate with two groups of host plants, temperate legumes and tropical legumes, forming indeterminate nodule and determinate nodules respectively. The indeterminate nodules are those where the rhizobia do not infect all the cells which form the nodule whereas all the cells forming the determinate nodules are infected by the rhizobia.

3.1.3 Early Nodulin (ENOD) gene expression in host plant

Certain host genes involved in nodule formation are expressed in the cortical cells which form the primordia and their expression is induced by Nod factors. These include ENOD12, Gm93, ENOD40 and MtPRP4 which can be induced when the root is inoculated with only Nod factors, unlike ENOD5, which is also expressed in the primordia but only when rhizobia are present and the cell comes in direct contact with the Nod factors (Heidstra *et al.,* 1997). The genes such as ENOD12 and ENOD5 are also activated

in the epidermis, where cells come into direct contact with Nod factors. ENOD12 and ENOD40, however, seem to be receiving a signal to cause induction of expression as direct contact of Nod factors is not required by the cell for expression to occur. The expression of ENOD40 occurs in the pericycle and dividing cortical cells and is one of the first genes to be induced. It encodes a short peptide thought to control the dedifferentiation of cells in the root cortex, therefore, it could be used as a marker of nodulation.

The first point of contact the host has with Nod factors is at the root hairs, which triggers responses at the root epidermis, but also in the cortex and pericycle. There are two possibilities as to how the Nod factors are able to elicit this response; either a secondary signalling molecule is used to transport the response, or Nod factors themselves are transported into the inner root and recognised there. The expression of ENOD12 and ENOD5 can be considered here as both are expressed at the epidermis where the cells are in direct contact with Nod factors. ENOD12 is also expressed in the nodule primordia before invasion by rhizobia, this would suggest the Nod factors are being transported, or a secondary signal is being produced to initiate its expression. ENOD5, however, is not expressed in the cortex until the cells come into contact with rhizobia. In indeterminate nodules, where not all cells are invaded by rhizobia, it is only in those which are invaded by the bacteria, and come in direct contact with the Nod factor, that express ENOD5. The fact that ENOD5 is not expressed until rhizobia are present suggests that the Nod factors themselves are not transported into the cortex to initiate ENOD12 expression and cortical division. A secondary messenger produced at the epidermis could be used, which is still to be determined (Heidstra and Bisseling, 1996).

3.1.4 Nod Factor perception

The actual mechanism of Nod factor perception is not clearly understood; however, possible genes and proteins have been suggested to be involved in the process (Guerts *et al.*, 1997; Ardourel *et al.*, 1994). There are a number of mechanisms that may be used to initiate the infection process. One being a receptor with varying affinities to different Nod factors, depending on their certain decoration due to varying genotypes. Specifically modified Nod factors could be more compatible with certain hosts due to a strong affinity, which will allow the specific infection response to be induced. Alternatively, two or more receptors could be present: the first being the entry receptor which is responsible for initiating the infection process by causing root hair deformation, and gene expression. A further receptor, an uptake receptor, could also be present to provide secondary signals to cause the formation of the infection threads and cortical cell division (Ardourel *et al.*, 1994).

Ardourel *et al.* (1994) were the first to suggest the hypothesis that two or more receptors are used in perception. This would fit the evidence that Nod factors which have many different modifications to the non-reducing end are able to cause root hair deformation, expression of ENOD12 and membrane depolarisation. The first stage, therefore, is not very specific and a wide range of Nod factors can elicit the response, but for the infection process to be completed only specific Nod factors are compatible. Ardourel *et al.* (1994) hypothesise that the first receptor would be the signalling receptor which is specific for a wider range of Nod factors than the second receptor, the uptake receptor, which is more

specific and allows only a small range of Nod factors to complete the infection process.

3.1.5 NodC gene homologues

Clearly *nod*C, as one of the common genes found in all rhizobia, has a vital role in the biosynthesis of Nod factors. Homologues to NodC also have been observed in yeast, mice, zebrafish and *Xenopus* and these homologues are believed be involved in similar signalling processes during development, and thought to catalyse the synthesis of chitooligosaccharides during a specific window in embryo development (Rosa *et al.*, 1988; Semino *et al.*, 1996). The homologue found in *Xenopus laevis*, known as DG42, is believed to have a role in the development of the embryo and its expression can only be detected between the midblastula and neurulation stages (Rosa *et al.*, 1988). The DG42 amino acid sequence demonstrates significant homology to the NodC amino acid sequence, *Streptococcus* hyaluronan synthase and fungal chitin synthases, although its exact biochemical function is still under debate. It is well accepted that NodC is a _ 1-4 N-acetylglucosaminyl transferase involved in the formation of chitooligosaccharides (DÈnarie and Debelle, 1996), but whether DG42 gene is responsible for the similar synthesis of chitooligomers, used as signalling molecules as in rhizobia, or hyaluronan, a major constituent of most vertebrate tissue and organ extracellular matrix is still to be determined. Hyaluronan is composed of alternating units of _ -1-4-linked N-acetylglucosamine and _-1-3-linked glucoronic acid and is also believed to have a role during embryo development in cell migration, proliferation and differentiation (Spicer *et al.*, 1997). Both HA and chitin have been produced as a result of over-expression of DG42 in a heterologous system (Semino *et al.*, 1996).

Evidence to show that the DG42 is a synthase of HA was presented by Meyer and Kreil (1996) who showed that rabbit kidney and human osteosarcoma expressing *Xenopus* DG42 produced an increased amount of HA. DeAngelis and Achyuthan (1996) have also demonstrated that the DG42 protein can synthesize HA in *Saccharomyces cerevisiae*, which do not naturally produce HA. From this they have concluded that DG42 is a membrane associated HA synthase and not a chitin synthase.

Evidence for DG42 as a synthase of chitooligosaccharides not HA was shown by Semino *et al.* (1996). They demonstrated that the synthesis of a DG42 homologue in zebrafish follows the same expression pattern as seen in *Xenopus* and that the chitooligosaccharide synthase activity can be disrupted by a DG42 antibody, whereas the synthesis of HA was not affected. Bakkers *et al.* (1997) also demonstrated that carp and zebrafish also produce chitooligosaccharides at specific intervals during embryo development. The production of the oligosaccharides could be blocked by antisera to DG42 and by doing so cause defects in the developing embryo. Recent analysis into the mechanism of chain elongation of chitooligosaccharides has shown that *in vitro* elongation by NodC and DG42-dependant elongation in zebrafish embryos is achieved by addition of the N-acetylglucosamine residue to the non-reducing terminal residue (Kamst *et al.*,1999). The discovery that HA elongation occurs at the reducing terminal residue (Pehm 1983), lead Kamst *et al.* (1999) to conclude that DG42 is indeed a chitin oligosaccharide synthase. This turn suggests that chitooligosaccharides may have an essential role in embryo development and this mechanism could possibly be widespread (Bakkers *et al.*, 1997).

The many homologues to nodulation genes suggests that these morphogens may be

involved in embryo development or organogenesis in numerous organisms. Nod factors may even be a universal control system (Schmidt *et al.*, 1994). The clarification of the role of DG42 within the *Xenopus* embryo will indicate whether similar chitoologosaccharides are required for embryo development in *Xenopus* as seen in nodulation. If these lipochitooligosaccharides are established as important signalling molecules for development within the *Xenopus* and other vertebrates, then other species such as the gall forming cynipid wasps could also use such morphogens to alter the developmental programme of their host.

Results

3.2 The search for *nodC* homologues in cynipid wasps

Nod C was chosen as our sequence to search for, as it is common to all rhizobia, is involved in the synthesis of the Nod factor backbone and homologues are known in many organisms. A NodC homologue found in *Xenopus laevis*, DG42, was also used to base primers on as both *Xenopus* and cynipids are eukaryotes. A PCR-based search was used with degenerate primers created from the consensus amino acid sequence of NodC and DG42. The primers were non-specific due to the degeneracy of the genetic code, therefore, two sets of primers were used in a nested PCR to increase the specificity of the final product. Nested PCR involves amplification with the outer set of primers and using this PCR product as a template for a second amplification with the inner primers. By carrying out nested PCR, the specificity of the amplified product by the degenerate primers is increased, and the homologous sequences within the wasp genome should be amplified. This work has been published in Schönrogge *et al.* (1998), which can be found

in the appendix.

To amplify any *nodC* or DG42 sequences from the cynipid genome, a specific PCR programme and reaction mix needs to be optimised. The primers must be carefully considered and parameters such as annealing temperature, template concentration and magnesium concentration optimised when developing a specific PCR reaction. Experiments to achieve this are presented below.

3.3 Selection of degenerate primers from consensus regions of NodC and DG42 for nested PCR

Degenerate primers, to be used in the PCR amplification of *nodC* and DG42 homologues, were decoded from the respective amino acid sequences encoded by these two genes. Two sets (four primers) were constructed, two outer and two inner primers for each gene. A NodC amino acid consensus sequence from several *rhizobia* was used to create the *nodC* primers, and an alignment of several NodC, chitin synthases and DG42 amino acid sequences was used to create the DG42 primer sequences (both provided by Alan Downie). Conserved regions of 6 or 7 amino acids were chosen as primer sites from the sequences, shown in Figure 3.1.A and B. 5'and 3' outer regions were chosen from NodC consensus sequence and the DG42 aligned sequence. 5' and 3' regions inner to those were chosen for the inner primers. The amino acid sequences were "reverse translated" into the respective codons giving several combinations for each primer. The complements for the 3' primer sequence were determined and turned backwards to give the correct orientation of the oligonucleotides.

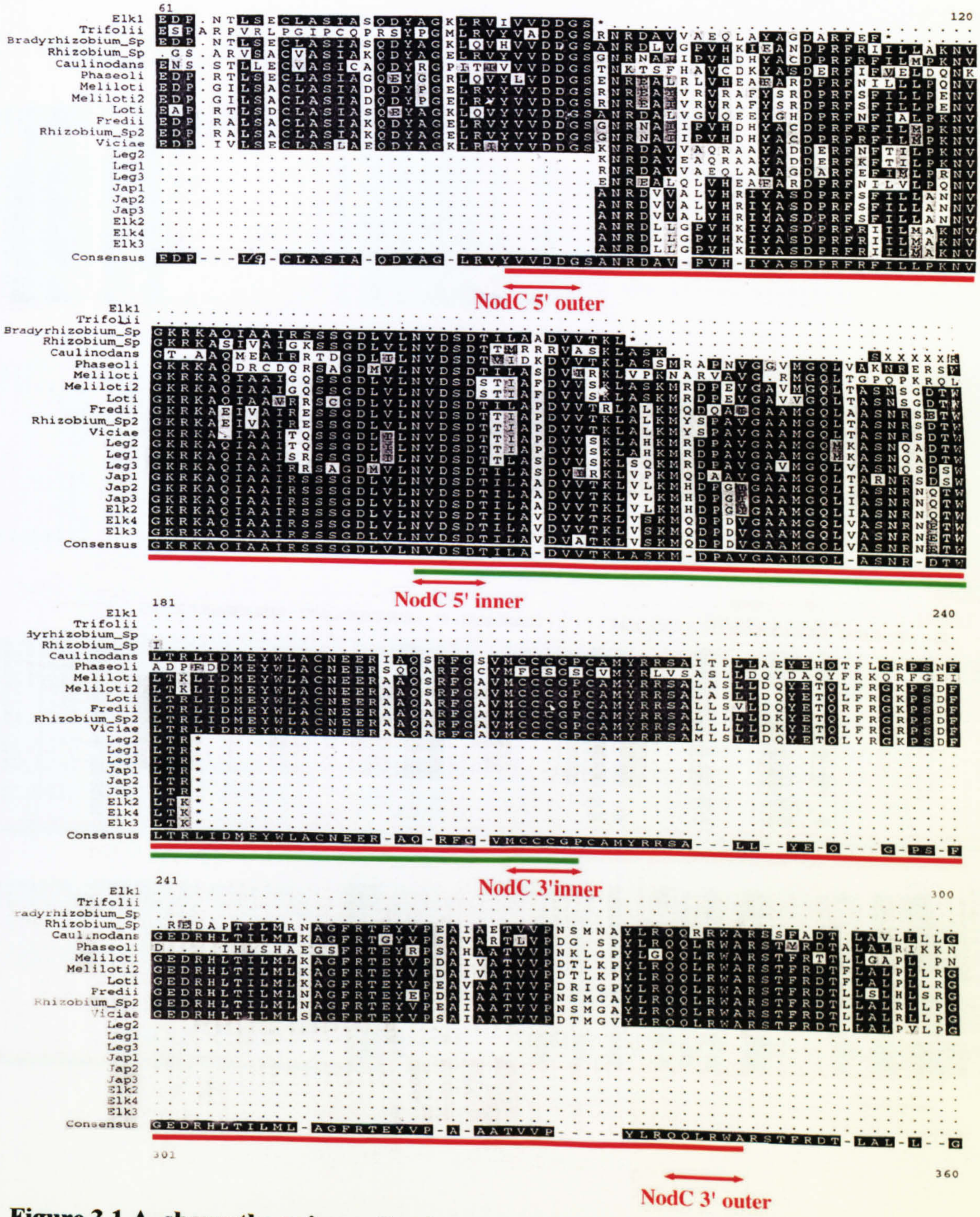


Figure 3.1.A. shows the primer sites for the *nodC* outer and inner primers on NodC amino acid consensus sequence. The dots indicate indentity and the dashed indicate gaps.

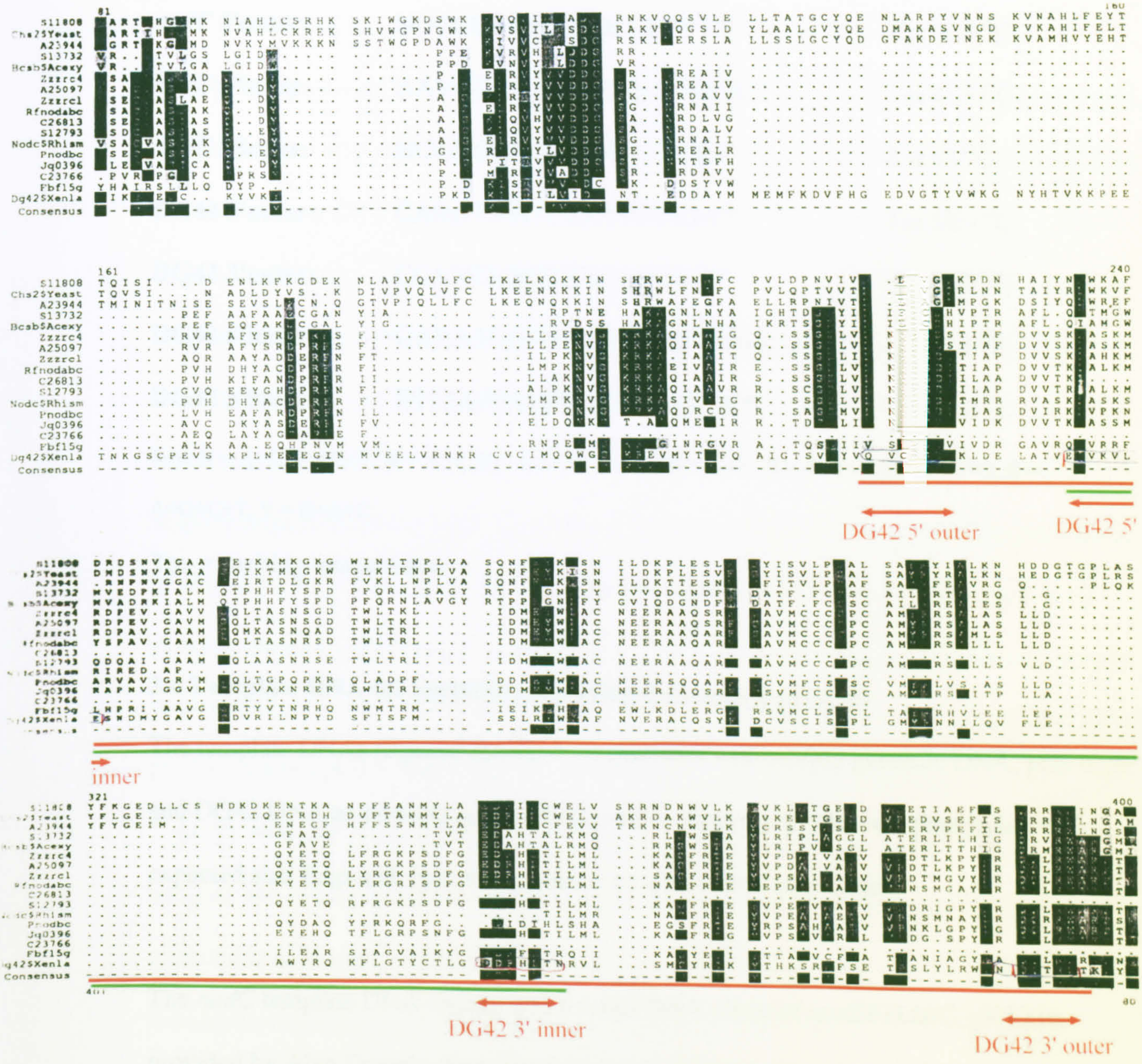


Figure 3.1.B. shows the primer sites for the DG42 outer and inner primers on DG42 amino acid sequence. . The dots indicate identity and the dashed indicate gaps.

The sets of oligonucleotides sent for synthesis were:

***nodC* 5'outer:**	TAYGTNGTNGAYGAYGGN	Tm 51.1 ^{O}C
***nodC* 3'outer:**	NGCCCANCKNARYTGYTG	Tm 56.9 ^{O}C
***nodC* 5'inner:**	AAYGTNGAYWSNGAYACN	Tm 45.8 ^{O}C
***nodC* 3'inner:**	NGGNCCRCARCARCACAT	Tm 57 ^{O}C
DG42 5'outer:	CARGTNTGYGAYWSNGAY	Tm 50.9 ^{O}C
DG42 3'outer:	CCANCKNGTYTGYTGRTT	Tm 46.9 ^{O}C
DG42 5'inner:	GARATGGTNAARGTNYTNGAR	Tm 51.5 ^{O}C
DG42 3'inner:	RTTNGTNARRTGNCTRTCRTC	Tm 52.8 ^{O}C

Key: R = A+T, Y = C+T, M = A+C, K = G+T, S = G+C, W = A+T, H = A+T+C, B = G+T+C, N = A+G+C+T, V = G+A+C

Tm = melting temperature.

3.4 Template DNA for amplification using degenerate primers

The template DNAs required for the PCR reactions was cynipid genomic DNA, plus *nodC* and DG42 DNA as controls to ensure the newly created primers were amplifying the expected size fragment from the sequence on which they were based.

The *nodC* template DNA chosen was a 6.6kb DNA clone of *nodEFDABC*, pKT230, provided by Alan Downie from The John Innes Institute, Norwich (Rossen *et al.*, 1984). *E.coli* cells carrying this plasmid, which I named pC98, were grown up on LB kanamycin plates and the plasmid purified using the "Quiglie" purification method (section

2.2.1.1(i)).

The DG42 template DNA was provided in a plasmid, pC4202, containing the whole DG42 cDNA by Prof. Igor Dawid from the Laboratory of Molecular Genetics, National Institute of Health, Maryland (Rosa *et al.*, 1988). I named the plasmid pC105.

The cynipid gall wasp template DNA was *A.quercuscalicis* genomic DNA, extracted from developing larva by first homogenising the larva and extracting the DNA using the boiling method (section 2.2.1.1(iii)).

3.5 Optimisation of PCR reaction mix using degenerate primers

3.5.1 Verification of PCR reaction mix

To test the PCR reaction system, existing Cytochrome b primers (CB1 and CB2) known to amplify sequences from cynipid genomic DNA were first used. It was possible, therefore, to demonstrate that the PCR reaction mixture and programme were working even if no results were obtained when the new sets of primers were used. The CB1 and CB2 primers were provided by Dr James Cook together with *Andricus californicus* template DNA and a programme which was optimised for the primers: 1 cycle at 94°C for 4 min, 40 cycles of: 1 min at 94°C and 1 min 42°C and 2 min 72°C, 1 cycle at 72°C for 10 min. This programme was used as the basic programme at the start. The primers did amplify the expected size product from the *Andricus californicus* template (not shown), demonstrating the reaction mix and PCR programme were working. This combination was used alongside the NodC and DG42 primer-template combinations while optimisation was being carried out to ensure the reaction mix was working.

3.5.1 Determination of magnesium concentration for optimal PCR products

The concentration of $MgCl_2$ in the reaction mix can affect the specificity of the amplification so it is important to find the optimal concentration for the primers. At high concentrations, $MgCl_2$ may prevent complete denaturation of the product at 94°C stage of each cycle, reducing the total yield. If the concentration of $MgCl_2$ is too low then the extension reaction may be impaired as it is required as a co-factor by DNA polymerase.

To optimise the amplification by *nodC* and DG42 primers on the *nodC*, DG42 and cynipid templates the $MgCl_2$ was tested at 0.5mM, 1mM and 2mM concentrations for all primer-template combinations. The specificity of the amplified products was determined by the amount of background smearing observed behind the bands when run on an agarose gel. The highest yield was obtained when using a 1mM concentration (results not shown), and this was used for the ensuing reactions.

3.5.2 Optimisation of annealing temperature to improve amplification of specific PCR products

The annealing temperature affects the binding of the primers to the template. If the temperature is too low then non-specific binding will occur between the template and the primers, increasing the amplification of non-specific product. A high temperature may prevent annealing altogether, and reduce the amount of PCR product. To optimise the annealing temperature for my PCR programme, the basic programme was run with annealing temperatures of 42°C, 48°C, 50°C and 55°C, using the usual combinations of DNA template and outer primers. The most specific yield was obtained using 48°C (results not shown), giving the programme: 1 cycle at 94°C for 4 min, 40 cycles of: 1 min

at 94°C and 1 min 48°C and 2 min 72°C, 1 cycle at 72°C for 10 min.

3.5.3 Optimisation of elongation time to reduce non-specific amplification

The time at each stage can also affect specificity of the amplification. Taq polymerase can produce 1kb in 30 sec therefore the 2 min elongation stage used in the basic programme may allow non-specific sequences to be amplified. The time was reduced to 1 min, however, little difference was observed. The shorter time was kept as it reduced the overall length of the PCR programme.

3.5.4 The development of the split programme using optimal parameters

Although 48°C gave the most specific product, the yield was low. To increase the yield of product while keeping the specificity high, a split programme with two different annealing temperatures was developed. A low temperature for the first five cycles allows the primers to bind readily to the template and a high temperature increases the specificity of the reaction. The programme: 4 min at 94°C for 1 cycle; 60 sec at 94°C, 60 sec at 42°C, 60 sec at 72°C, for 5 cycles; 60 sec at 94°C, 60 sec at 48°C, 60 sec at 72°C, for 35 cycles; 5 min at 72°C for 1 cycle. Thus the five cycles at low annealing temperature should allow bulking up template and the remaining cycles at high annealing temperature increase the specificity. This was used as the final, optimised programme, producing the highest and most specific yield.

3.6 PCR products from optimised PCR programme

The predicted sizes for the outer and inner primers on each template is shown in Table 3.1 below. The DG42 gene contains four introns, two of which are positioned between the

outer primer sites. cDNA was used for DG42 template DNA in the PCR reactions, therefore, predicted sizes without introns are given and the size including introns is given in brackets.

Table 3.1 The predicted sizes of PCR product from the outer and inner primers on the NodC and DG42 template

Primer template combination	Predicted size amplified by outer primers (including introns)	Predicted size amplified by inner primers (including introns)
NodC primers of *nodC* DNA template	597bp	219bp
DG42 primers on DG42 cDNA template	438bp (871bp)	288bp (397bp)

The PCR products amplified in the first step of the nested PCR programme by the *nodC* and DG42 outer primers on the *nodC*, DG42 and cynipid templates are shown in Figure 3.2. The PCR products are mainly smears although in lane 2, a 600bp band can be seen, amplified by *nodC* primers on nodC template. Lane 3 shows a concentration of smearing around 600bp for *nodC* primers on *A.quercuscalicis* template. Smears are obtained as the primers used to amplify from the template are degenerate, varying at one or a number of bases, and therefore give a smear of different sized amplified products.

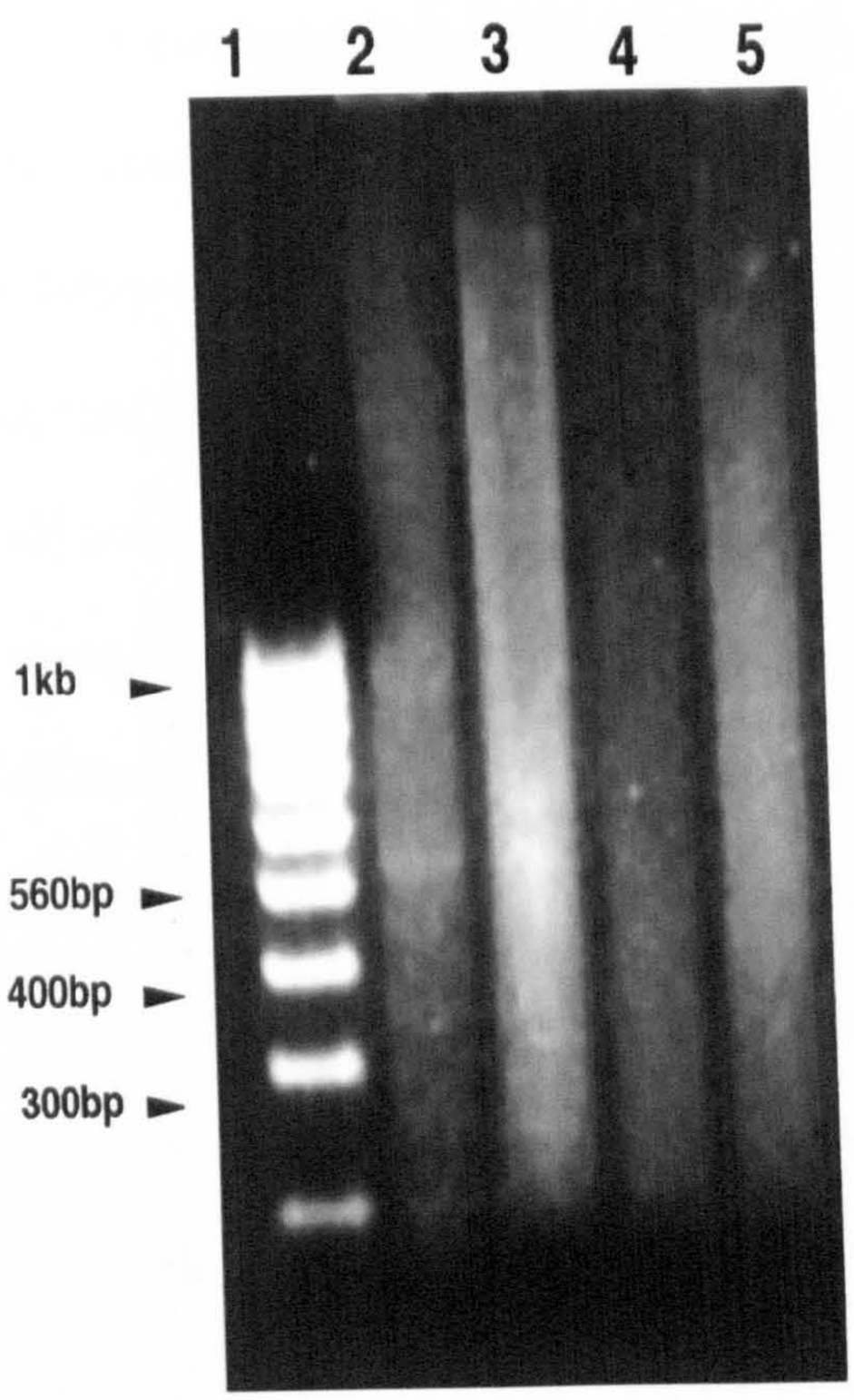


Figure 3.2 Agarose gel (1.2%) of PCR products amplified from the outer PCR reaction. Lane 1 contains 100bp size marker. Lane 2 contains products amplified by *nodC* outer primers on *nodC* template DNA. Lane 3 contains products amplified by *nodC* outer primers on *A.quercuscalicis* template DNA. Lane 4 contains product amplified by DG42 outer primers on DG42 template DNA. Lane 5 contains product amplified by DG42 outer primers on *A.quercuscalicis* template DNA.

These PCR products, used as templates for amplification by the inner primers, should increase specificity enabling a specific template to be amplified, which contains both the outer and inner primer sites. Figure 3.3. shows the nested PCR products, in lane 2 the 535bp and 425bp products amplified by *nodC* inner primers on *nodC* template. Surprisingly, the expected size fragment of 219bp did not appear to be amplified. It may have been present at a low concentration which was unable to be seen on the gel or may not have been amplified at all. The fragment amplified by DG42 inner primers on DG42 template, lane 4, was larger than the expected size giving a fragment of 375bp, however, this was the only fragment amplified by these primers suggesting the fragment may be specific. The expected size of the fragments for the *A.quercuscalicis* template is unknown as the presence and size of introns has not been determined. The amplified product by the *nodC* inner primers on the *A.quercuscalicis* template was 425bp, lane 3. The DG42 inner primers amplified products of 425bp, 500bp and 1kb from the *A.quercuscalicis* template, shown in lane 5. The PCR products obtained from the nested reaction using the optimised programme compared to the expected results are summarised in Table 3.2. The two different sets of primers both amplified the same size fragment from the *A.quercuscalicis* genomic DNA, suggesting that the 425bp fragment may be the homologue for which we are searching.

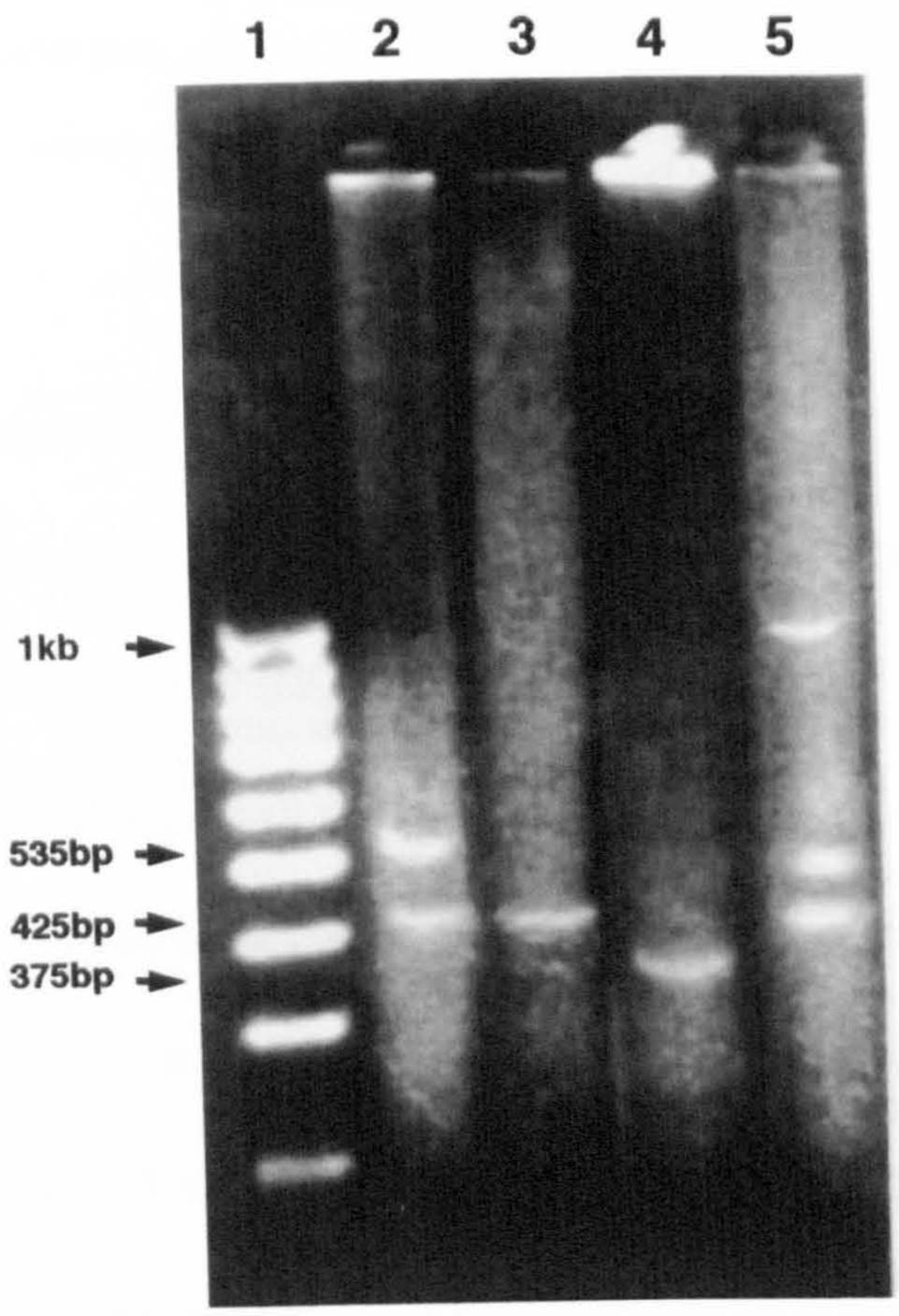


Figure 3.3 Agarose gel (1.2%) of PCR products amplified from the nested PCR reaction. Lane 1 contains 100bp size marker. Lane 2 contains 535bp and 425bp products amplified by *nodC* primers from *nodC* template DNA. Lane 3 contains the 425bp product amplified by *nodC* primers from *A.quercuscalicis* template DNA. Lane 4 contans the 375bp product amplified by DG42 primers from DG42 template DNA. Lane 5 contains 1kb, 500bp, 425bp products amplified by DG42 primers from *A.quercuscalicis* template DNA.

Table 3.2 A table comparing the predicted and the observed nested PCR products from the different primer-template combinations.

Primer template combination	Predicted size of amplified product	Size of product obtained
nodC primers and *nodC* DNA	219bp	535bp and 425bp
nodC primers and *A.quercuscalicis* genomic DNA	Unknown	425bp
DG42 primers and DG42 cDNA	288bp	375bp
DG42 primers and *A.quercuscalicis* genomic DNA	Unknown	1kb, 500bp and 425bp

3.6.1 Verification of PCR product

The DG42 DNA template used in the PCR reaction was the pC105 plasmid containing the cDNA of DG42. The DG42 fragment would therefore be present in high copy numbers, compared to that if total genomic DNA was being used. To determine if the PCR product could be amplified when the primer target sequences were present in low copy numbers, as would be expected if total genomic DNA was being used as template DNA, varying concentrations of pC105 were used as template DNA. The PCR products were amplified from as little as 2.5pg template DNA confirming the fragment could be amplified if present at a low copy number.

To ensure this was the correct fragment being amplified by DG42 primers on the DG42 template, Southern blotting was used, probing the PCR products with the purified DG42 fragment from the pC105 plasmid. PCR products amplified by DG42 nested primers were run on a 1.2 % agarose gel, transferred onto Hybond N (Amersham) membrane, using the standard Southern blotting protocol (section 2.2.2.1). The membrane was subjected to

pre-hybridisation and hybridised using ^{32}P labelled DG42 fragment. The blot was then washed using a stringent SDS and SSC solution and exposed to Fuji X-ray film overnight. The hybridisation of the purified DG42 fragment to the PCR product (results not shown), confirmed that the correct product was being amplified by the primers.

3.6.2 Hybridisation of DG42 fragment to PCR products

The purified DG42 fragment from pC105 hybridises to the PCR product amplified by the DG42 primers on the DG42 template. To determine if the PCR products amplified by the *nodC* primers on the *nodC* and *A.quercuscalicis* templates, and the product amplified by the DG42 primers on the *A.quercuscalicis* template, are recognised by the DG42 fragment from pC105, the PCR products were probed with the purified DG42 fragment. To achieve this the pC105 plasmid, the purified DG42 fragment from pC105, and the PCR products from nodC and DG42 primers were run on an agarose gel. Figure 3.4 Shows the agarose gel (1.2%) and Southern blot of the products amplified by the *nodC* and DG42 primers on the *nodC*, DG42 and *A.quercuscalicis* templates. The Southern blot shows that the pC105 plasmid does hybridise to the purified DG42 fragment as expected, shown in lane 3 and 4. The purified DG42 fragment in lane 5 gives an intense signal confirming the identity and efficiency of the probe. Lane 6 shows a weak signal at 375bp, indicating the hybridisation of DG42

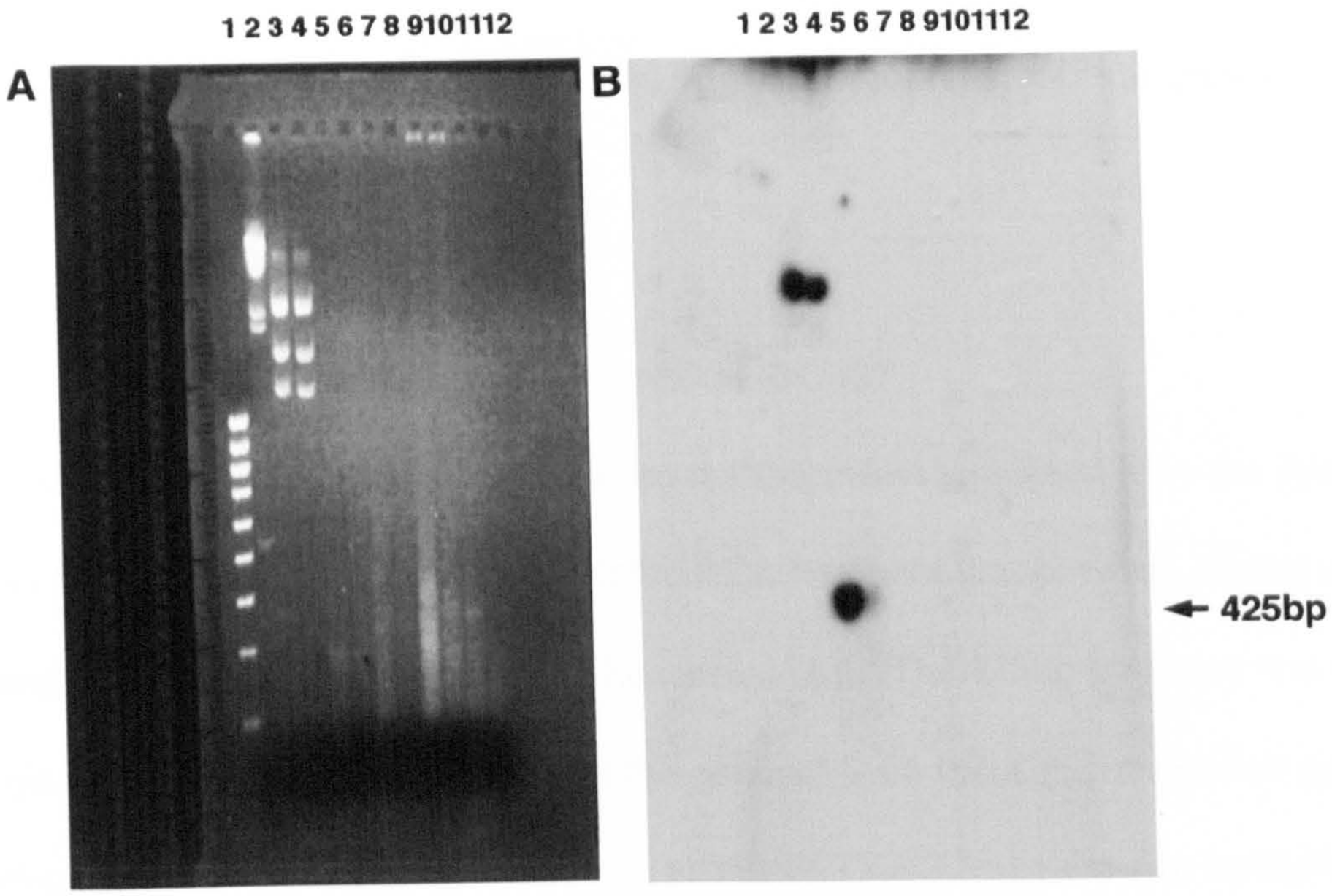


Figure 3.4 (A) Agarose gel (1.2%) and (B) Southern blot showing PCR products amplified by both *nodC* and DG42 inner primers on *A.quercuscalicis*, DG42 and *nodC* templates. In B, products were probed with purified DG42 fragment from pC105, to determine if any homology exists between DG42 and the *nodC* primer products. Lane 1 contains 100bp size marker. Lane 2 Lambda HindIII size marker. Lane 3 undigested pC105. Lane 4 undigested pC105. Lane 5 purified DG42 fragment from pC105. Lane 6 PCR product amplified by DG42 inner primers on DG42 template. Lane 7 PCR product from *nodC* inner primers and *nodC* template DNA. Lane 8 PCR products from *nodC* inner primers and *nodC* template DNA. Lane 9 PCR products from DG42 outer primers and *A.quercuscalicis* template DNA. Lane 10 PCR product from *nodC* outer primers and *A.quercuscalicis* template DNA. Lane 11 PCR product from DG42 inner primers and *A.quercuscalicis* template DNA. Lane 12 PCR products from *nodC* inner primers and *A.quercuscalicis* template DNA. primers on *nodC* and *A.quercuscalicis* templates, and DG42 primers on *A.quercuscalicis* templates do not show hybridisation.

probe to the PCR product amplified by the DG42 inner primers on the DG42 template. The other products in lanes 7-12 amplified by *nodC* primers on *nodC* and *A.quercuscalicis* templates, and DG42 primers on *A.quercuscalicis* templates do not show hybridisation. This, however, does not indicate that no homology exists, just that it is not great enough to hybridise to the probe.

3.7 Is the PCR product a homologue of NodC or DG42?

To determine if homology does exists between the fragments amplified from the cynipid DNA and DG42 or *nodC* sequences, the 425bp PCR fragment was purified, cloned and sequenced, to enable sequence analysis to be carried out. The 425bp fragment was used as both *nodC* and DG42 primers amplified this product from the *A.quercuscalicis* genomic DNA template, and was of similar size to the predicted DG42 fragment size including introns.

3.7.1 Cloning the 425bp PCR product produced using DG42 primers on gall wasp DNA

The 425bp product was first purified using a Quiagen PCR cleanup column (section 2.2.3.1). This removes unused primers, dNTPs, Taq polymerase, $MgCl_2$ and PCR buffer, leaving the amplified PCR product. The Taq polymerase adds a single deoxyadenosine to the 3'- end of the amplified fragment and this was used to ligate the product into a specially designed Promega pGEM™-T Easy vector (shown in Figure 2.3) which has 3'T overhangs at the insertion site, increasing the efficiency of ligation to PCR products. The vector also contains the multiple cloning site within the lacZ gene encoding for β-galactosidase, which is flanked by the T7 and SP6 RNA polymerase promoters. Insertion

into lacZ inactivates β-galactosidase production and allows colour screening of colonies on IPTG X-gal plates. The purified PCR product was ligated to the pGEM™-T Easy vector overnight at 14^0C and transfected into competent DH 5 α *E.coli* cells using electroporation (section 2.2.3.3). The ligation and the controls were plated out on LB Amp-IPTG-X-gal plates and positive clones were identified using the blue/white screening. The IPTG induces the expression of β galactosidase and, if active, this metabolises the X-gal to produce a blue colony. The positive clones appear white, due to the inactivation of β galactosidase by the insert preventing the cell from metabolising X-gal. 36 positive colonies were picked and grown up overnight in LB-amp media. Plasmid DNA extractions were carried out on the overnight cultures and the plasmid DNA was digested with EcoR1 to cut out the insert (section 2.2.3.5). Five of the digests contained inserts, however, only one was the expected size.

3.7.2 Verification of cloned PCR products by Southern blotting

To determine if the clone of the 425bp insert (clone 1) was the purified PCR product, a Southern blot was carried out. The original 425bp PCR product and all the five cloned fragments were probed with clone 1. The five positive clones and the original PCR product were run on a 1.2% agarose gel, blotted onto Hybond N (Amersham) using the standard Southern blotting protocol and subjected to pre-hybridisation treatment before hybridisation with ^{32}P labelled plasmid DNA of clone1. Figure 3.5 shows the agarose gel of the five digested plasmids containing inserts and the Southern blot when probed with clone 1. The probe hybridised with the original PCR product used as the insert and clone 1, confirming clone 1 contains the correct 425bp insert. The other inserts have not hybridised demonstrating that these are not the PCR fragment insert.

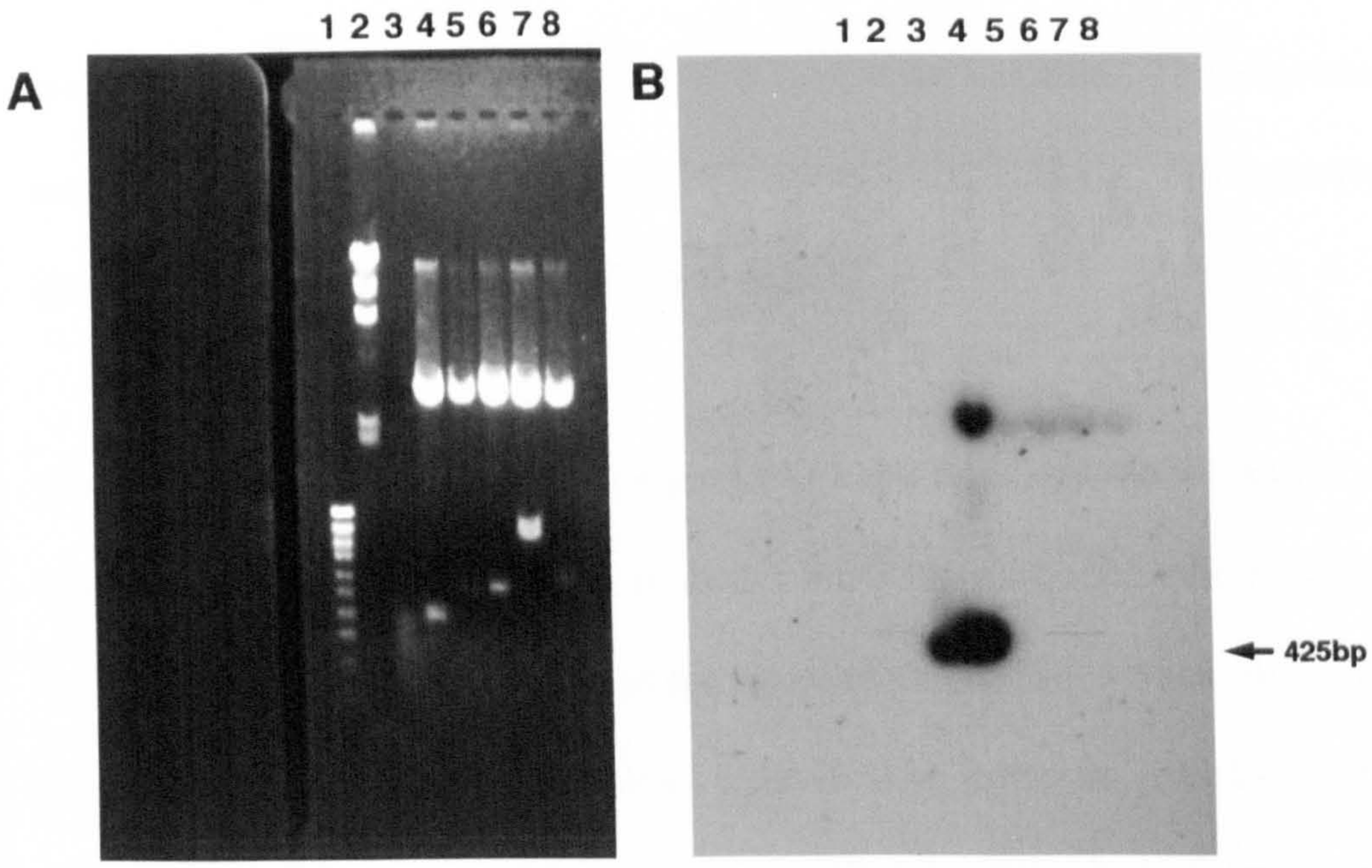


Figure 3.5 (A) Agarose gel (1.2%) and (B) Southern blot of possible clones of purified PCR product from DG42 nested primers on *A.quercuscalicis*. In B, clones were probed with purified insert of clone 1. Lane 1 100bp size marker. Lane 2 Lambda HindIII size marker. Lane 3 Purified PCR product from DG42 nested primers. Lane 4 Clone 1. Lane 5 Clone 2. Lane 6 Clone 3. Lane 7 Clone 4. Lane 8 Clone 5.

3.7.3 Sequence analysis of the putative nodC/DG42 homologue

The cloned PCR product was sent to be sequenced at the ABC sequencing facility, Imperial College School of Medicine, Charing Cross hospital. Sequence analysis was carried out using BLAST (Altschul, 1990). The sequence was first translated into the six possible reading frames to ensure the presence of the primer sequence at either end, shown in Figure 3.6. The six possible reading frames were then used in a Blastx search, comparing the six possible reading frames to all amino acid databases for homology, as shown in Figure 3.7. Significant homologies were obtained to two proteins hypothetical protein from *Caenorhabditis elegans* with no known function. The alignment to these sequences show 53% and 43% positive homology respectively. No homology to any chitin synthase was found in the library search.

Comparison of the PCR product sequence and the NodC and DG42 amino acid sequences was therefore carried out directly to determine if there was some homology. ClustalW was used for multiple alignment of the six possible reading frames shown in Figure 3.8. NodC and DG42 did show homology to each other, however, little homology was found between the PCR amino acid sequences and NodC or DG42. In frame 5 the primer sequence sites show homology, in particular the 3' sequence, which would explain the amplification by PCR despite the lack of internal homology. Frame 5 showed the greatest homology, therefore, was aligned with DG42 and NodC, shown in Figure 3.9. Homology at the primer sites at each end of the sequence can be seen (indicated by a *), and some internal homology exists, however, this is not significant enough to suggest gene homology.

A

TGAAATGGTGAAGGTGCTCGAGAAAATGATGAAAATACCATGGCGATGAAA
ATGCCGTCAATCAACTTTGAGTTCACCACGATCAACGATACAAAAAATGCAA
AAGGAATCTACATAATGAGCCCTTGCCATAATCCTGAGTGAACGAGTTGAGT
ATTTGTAAAGAAGAATACTGGAATTTTTAGTATATTTTTCAAAATCACTATTT
AAGATAGTGAAAGTCAATAGCTGGCTACTGACTTACTTTTCACTGTCAATTAA
ATATTACATACTTCGTATTTTTCGATATTTATCTGATAATTTAGATGCTTCAAC
TAAAAGTAGAAGGTATGTAAATATTCAAGACGACCGACATTTAACAAATA

B

<u>5'3' Frame 1</u>

-NGEGARENDENTMAMK.MPSINFEFTTINDTK.NAK.GIYIMSPCHNPE-TS-
VFVK.KNTGIFSIFFKITI-DSESQ-LATDLLFTVN-ILHTSYFSIFI--FRCFN-K.-
K.VCK.YSRRPTFNK.

<u>5'3' Frame 2</u>

EMVK.VLEKMMMKIPWR-K.CRQSTLSSPRSTIQKMQKEST--ALAIILSERVEYL-
RRILEFLVYFSK.SLFK.IVK.VNSWLLTYFSLSIK.YYILRIFRYLSDNLDASTK.SRRY
VNIQDDRHLTN

<u>5'3' Frame 3</u>

K.W-RCSRK.--K.YHGDENAVNQL-VHHDQRYYK.CK.RNLHNEPLP-S-
VNELSICKEEYWNF-YIFQNHYLR--K.SIAGY-LTFHCQLNITYFVFFDIYLII-
MLQLK.VEGM-IFK.TTDI-QI

<u>3'5' Frame 1</u>

YLLNVGRLEYLHTFYF-LK.HLNYQINIEK.YEVCNI-LTVK.SKSVASY-LSLS-
IVILK.NILK.IPVFFFTNTQLVHSGLWQGLIM-IPFAFFVSLIVVNSKLIDG
IFIAMVFSSFSRAPSPF

<u>3'5' Frame 2</u>

IC-MSVVLNIYIPSTFS-SI-IIR-ISKNTK.YVIFN-Q-K.VSQ-PAIDFHYLK.--F-KIY
K.FQYSSLQILNSFTQDYGK.GSLCRFLLHFLYR-SW-TQS-LTAFSSPWYFHH
FLEHLHHF

<u>3'5' Frame 3</u>

FVK.CRSS-IFTYLLLLVEASKLSDK.YRK.IRSM-YLIDSEK.-VSSQLLTFTILNSDF
EK.YTKNSSILLYK.YSTRSLRIMARAHYVDSFCIFCIVDRGELKVD-R
HFHRHGIFIIFSSTFTIS

Figure 3.6 (A) Sequence of PCR product amplified from *A.quercuscalicis* DNA template by DG42 primers. (B) Six possible reading frames of the sequence.

BLAST

BLASTX 2.2.1 [Apr-13-2001]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs", Nucleic Acids Res. 25:3389-3402.

RID: 998819256-23003-24499

Query=
(365 letters)

Database: nr
743,070 sequences; 235,404,442 total letters

Sequences producing significant alignments:

	Score (bits)	E Value
gi|7496212|pir||T31922 hypothetical protein C17F4.4 - Caeno...	31	1.8
gi|14670164|gb|AAK72056.1|AF016655_8 (AF016655) Hypothetica...	31	1.8
gi|7504773|pir||T32116 hypothetical protein F59E11.13 - Cae...	30	3.0
gi|11499675|ref|NP_070917.1| conserved hypothetical protein...	29	6.8
gi|9631419|ref|NP_048327.1| ORF MSV256 hypothetical protein...	29	8.8

Alignments

>gi|7496212|pir||T31922 hypothetical protein C17F4.4 - Caenorhabditis elegans
Length = 347

Score = 31.2 bits (69), Expect = 1.8
Identities = 17/41 (41%), Positives = 22/41 (53%), Gaps = 4/41 (9%)
Frame = +2

```
Query: 191 FSKSLFKIVKVNSWLLTYFSLSIKYYILRIF----RYLSDN 301
           FS S+FK+ K   W   YFS+  KY+  R       R+L DN
Sbjct: 5   FSASIFKMKKSLFWNFFYFSIEQKYFSARFSPQNQRFLKDN 45
```

g|10864434|gb|AAG24147.1| (AF016685) Hypothetical protein F59E11.13 [Caenorhabditis elegans]
Length = 338

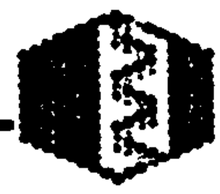
Score = 30.4 bits (67), Expect = 3.0
Identities = 20/62 (32%), Positives = 30/62 (48%)
Frame = +2

```
Query: 179 FLVYFSKSLFKIVKVNSWLLTYFSLSIKYYILRIFRYLSDNLDASTKSRRYVNIQDDRHL 358
           +L  FSK       + S+ L  F +S+KY+ L  F Y +D L    K+    N+  D+ L
Sbjct: 123 YLWMFSKLYLMFWVLGSFTLGLFLISLKYTTLGEFDYTTDQLTEEFKTN--YMLTNDQVL 180

Query: 359 TN 364
Sbjct:     N
```

Figure 3.7 Results from Blastx search using cynipid PCR product

EMBL
European Bioinformatics Institute



Your ClustalW Results:

```
DG42        TKLDELATVEMVKVLESNDMYGAVGGDVRILNPYDSFISFMSSLRYWMAFNVERACQSYF 300
5           ------------------------------------------------------------
3           ----------------------------------------------KW-RCSRK------- 7
1           ------------------------------------------------------------
2           ------------------------------------------------------------
4           ------------------------------------------------------------
6           ------------------------------------------------------------

NodC        GAVMCCCGPCAMYRRSALASLLDQYETQLFRGKPSDFGEDRHLTILMLKAGFRTEYVPDA 261
DG42        DCVSCISGPLGMYRNNILQVFLEAWYRQKFLGTYCTLGDDRHLTR-VLSMGYRTKYTHKS 359
5           -----IC----------MSVVLNIYIPSTFS-SI-IIR---------ISKNTKYVIFN-Q 33
3           ----------------KYHGDENAVNQL----------------VHHDQRYKKCKRN 32
1           ----------------NGEGARENDENTMAMKMPSIN---------FEFTTINDTKNAK 34
2           ----------------EMVKVLEKMMKIPWR----------------KCRQSTLSSPRS 27
4           ----------------YLLNVGRLEYLHTFYF--------------LKHLNYQINIEKYE 30
6           ----------------FVKCRSS-IFTYLLLLVEAS----------KLSDKYRKIRSM 31

NodC        IVATVVPDTLKPYLRQQLRWARSTFRDTFLALPLLRGLSPFLAFDAVGQNIGQLLLALSV 321
DG42        RAFSETPSLYLRWLNQQTRWTKSYFREWLYNAQWWHKHHIWMTYESVVSFIFPFFITATV 419
5           -KVSQ-PAIDFHYLK----F-KIY-KFQYSSLQILNSFTQDYGKGSLCRFLLHFLYR--- 82
3           LHNEPLP-S-VNELS---------ICKEEYWNF-YIFQNHYLR--KSIAGY-LTFHCQLN- 77
1           GIYIMSPCHNPE-TS--------VFVKKNTGIFSIFFKITI-DSESQ-LATDLLFTVN--- 81
2           TIQKMQKEST--ALAIILSERVEYL-RRILEFLVYFSKSLFKIVKVNSWLLTYFSLSIK- 83
4           VCNI-LTVKSKSVASY-LSLS-IVILKNILKIPVFFFTNTQLVHSGLWQGLIM-------- 80
6           -YLIDSEK-VSSQLLTFTILNSDFEKYTKNSSILLYKYSTRSLRIMARAHYVDS------- 83

NodC        VTGLAHLIMTATVP--WWTILIIACMTIIRCSVVALHARQLRFLGFVLHTPINLFLILPL 379
DG42        IR----LIYAGTIWNVVWLLLCIQIMSLFKSIYACWLRGNFIMLLMSLYSMLYMTGLLPS 475
5           ------------------------------------SW-TQS----LTAFSSPWYFHHFLEH 103
3           ----------------------------------------ITYFVFFDIYLII-MLQL 94
1           ----------------------------------------ILHTSYFSIFI--FRCFN 97
2           ----------------------------------------YYILRIFRYLSDNLDAST 101
4           ----------------------------------------IPFAFFVSLIVVNSKLID 98
6           ----------------------------------------FCIFCIVDRGELKVD-RHFH 102

NodC        KAYALCTLSNSDWLSR--------------YSAPEVPVSGGK-----------QTPIQT 413
DG42        KYFALLTLNKTGWGTSGRKKIVGNYMPILPLSIWAAVLCGGVGYSIYMDCQNDWSTPEKQ 535
5           LHHF------------------------------------------------------- 107
3           KVEGM-IFKTTDI-QI------------------------------------------- 108
1           -K-KVCKYSRRPTFNK------------------------------------------- 111
2           KSRRYVNIQDDRHLTN------------------------------------------- 117
4           GIFIAMVFSSFSRAPSPF----------------------------------------- 116
6           RHGIFIIFSSTFTIS-------------------------------------------- 117

NodC        SGRVTPDCTCSGE---------------------------------------- 426
DG42        KEMYHLLYGCVGYVMYWVIMAVMYWVWVKRCCRKRSQTVTLVHDIPDMCV 585
5           ------------------------------------------------
3           ------------------------------------------------
1           ------------------------------------------------
2           ------------------------------------------------
4           ------------------------------------------------
6           ------------------------------------------------
```

Figure 3.8 ClustalW amino acid sequence alignment of NodC, DG42 and 6 reading frames of PCR product.

Your Multiple Sequence Alignment:

382067.278128-176155.aln

```
CLUSTAL W (1.81) multiple sequence alignment


DG42            MKEKAAETMEIPEGIPKDLEPKHPTLWRIIYYSFGVVLLATITAAYVAEFQVLKHEAILF 60
NodC            ----------------------------------MTLLATTSIAAISLYAMLS------ 19
Aqc             ------------------------------------------------------------


DG42            SLGLYGLAMLLHLMMQSLFAFLEIRRVNKSELPCSFKKTVALTIAGYQENPEYLIKCLES 120
NodC            --TVYKSAQVFHARRT------TISTTPAKDIETNPVPSVDVIVPCFNEDPIVLSECLAS 71
Aqc             ------------------------------------------------------EMVKVLEK 8
                                                                       : : * .

DG42            CKYVKYPKDKLKIILVIDGNTEDDAYMMEMFKDVFHGEDVGTYVWKGNYHTVKKPEETNK 180
NodC            LAEQDYAG-KLRIYVVDDG-----------------------------------SKNR 93
Aqc             MMKIPWR---------------------------------------------------- 15
                     :

DG42            GSCPEVSKPLNEDEGINMVEELVRNKRCVCIMQQWGGKREVMYTAFQAIGTSVDYVQVCD 240
NodC            DAVVAQRAAYADDERFN----------FTILPKNVGKRKAIAAITQSSG---DLILNVD 139
Aqc             ------------------------------------------------------KCR 18


DG42            SDTKLDELATVEMVKVLESNDMYGAVGGDVRILNPYDSFISFMSSLRYWMAFNVERACQS 300
NodC            SDTTIAPDVVSKLAHKMR-DPAVGAAMGQMKASNQADTWLTRLIDMEYWLACNEERAAQA 198
Aqc             QSTLSSPRSTIQKMQKEST--ALAIILSER-----VEYL-RRILEFLVYFSKSLFKIVKV 70
                ..*      . :  :        .   .:       :     : .:  ::: .  :  :

DG42            YFDCVSCISGPLGMYRNNILQVFLEAWYRQKFLGTYCTLGDDRHLTNRVLSMGYRTKYTH 360
NodC            RFGAVMCCCGPCAMYRRSAMLSLLDQYETQLYRGKPSDFGEDRHLTILMLSAGFRTEYVP 258
Aqc             NSWLLTYFSLSIKYYILRIFRYLSDNLDASTKSRRYVNIQDDRHLTN------------- 117
                    :   . .   *    :  : :    .        : :*****

DG42            KSRAFSETPSLYLRWLNQQTRWTKSYFREWLYNAQWWHKHHIWMTYESVVSFIFPFFITA 420
NodC            SAIAATVVPDTMGVYLRQQLRWARSTFRDTLLALPVLPGLDRYLTLDAIGQNVGLLLLAL 318
Aqc             ------------------------------------------------------------


DG42            TVIR--LIYAGTIWMVVWLLLCIQIMSLFKSIYACWLRGNFIMLLMSLYSMLYMTGLLPS 478
NodC            SVLTGIGQFALTATLPWWTILVIGSMTLVRCSVAAYRARELRFLGFALHTLVWIFLLIPL 378
Aqc             ------------------------------------------------------------


DG42            KYFALLTLNKTGWGTSGRKKIVGNYMPILPLSIWAAVLCGGVGYSIYMDCQNDWSTPEKQ 538
NodC            KAYALCTLSNSDWLSRGSVAIAP----------------------TVGQQGATKMPG 413
Aqc             ------------------------------------------------------------


DG42            KEMYHLLYGCVGYVMYWVIMAVMYWVWVKRCCRKRSQTVTLVHDIPDMCV 588
NodC            RATSEIAYSGE---------------------------------------- 424
Aqc             ----------------------------------------------------
```

Figure 3.9 ClustalW amino acid sequence alignment of NodC, DG42 and frame 5.

3.7 Are cynipids capable of producing Nod factors?

From the experiments presented above, we can conclude that the 425bp PCR product amplified by the DG42 primers from the cynipid DNA is not significantly homologous to DG42 or NodC. There is homology at the primer sites and some internal homology, although not sufficient to suggest this is a chitin synthase. This does not mean that a *nodC* homologue does not exist, only it was not found here. The additional bands amplified by the *nodC* and DG42 primers in the PCR reaction, may show homology, although the products amplified by the *nodC* and DG42 primers are

of different sizes, suggesting that they are non-specific amplification. The 425bp product was amplified by both sets of primers on the *A.quercuscalicis* template, which is why this was chosen as a specific product to clone and sequence and the others thought to be non-specific amplification. Sequence analysis on the additional products needs to be carried out to confirm this.

The search for additional *nod* genes may help to further investigate and determine if *nod* genes are present in the gall genome. The presence of *nod* genes, such as nodL found in nematode, does not indicate that Nod factors are used, just that the organism may be capable of synthesising Nod factors or perhaps a similar oligosaccharide molecule. It remains possible that Nod factors or oligosaccharide signals are involved in gall induction and formation, however, to determine this, a bioassay specific to gall formation is required. For a specific bioassay, molecular markers, such as the induction of gall related genes or proteins are needed. The production of Nod factors by the larva also needs to be considered. Analysis of the larval extract and secretions are necessary to isolate the active molecules, although, gall related activity using a molecular bioassay is essential to first

determine activity, which can then be identified, be it Nod factor related or not. The development of a gall related bioassay is discussed in chapter 6.

4: Do inner-gall tissues express unique proteins ?

4.1 Protein Content of Inner-gall tissue.

Although cynipid galls have been the subject of research for hundreds of years, the mechanisms used by the cynipid wasp to redirect plant development remains unclear. The signal or set of signalling molecules produced are recognised by the plant and may even mimic existing plant signals. The elucidation of this will extend our understanding of plant development and the pathways involved in the tight control of organogenesis.

In addition to the visible developmental changes to plant tissue brought about by the cynipids, there are also changes in protein expression and in metabolite accumulation as discussed in chapter 1. These biochemical studies on gall tissue have been rather limited but have revealed high enzyme activity and high protein content in the inner-nutritive cells, and many important enzymes are produced and nutrient gradients form around the larva (Bronner, 1992).

Such changes must also be reflected in changes in gene expression, but little genetic analysis has been carried out on gall tissue. With the development of new technology and the high throughput analysis of genomics, transcriptomics and proteomics, it will soon be possible to identify at the molecular level how the plant responds to cynipid signals during gall formation. For example, to analyse differential gene expression, one effective approach would be to use cDNA/AFLP (cDNA/amplified fragment length polymorphism) as described by Bachem *et al.* (1996). This is a PCR based approach, amplifying

fragments from cDNA, providing a set of transcript-derived fragments (TDF) throughout development that would enable the identification of genes expressed at certain phases of gall development. This genomics approach to map the "transcriptome" would provide crucial information about gene expression in gall development and provide possible genetic markers that could be used in a bioassay. This is clearly a large-scale project such as those currently underway on industrial scales by biotechnology companies, and until very recently, the difficulties in preparation of oak DNA and RNA have precluded pilot experiments. Improved techniques have now overcome this hurdle and future projects will allow this approach.

Rather than analysing changes in gene expression, a more direct approach is to use 2-D gel electrophoresis to map and identify all of the proteins which are expressed at all stages of gall formation, a proteomics approach. In 2-D gel electrophoresis, proteins are separated according to molecular mass in one dimension, and then charge by isoelectric focusing, in the other dimension, so spreading them out as spots on a membrane. Comparing such 2-D arrays to equivalent ones of non-gall tissue throughout plant development, will reveal differences which can be analysed by protein sequence determination. Thus proteins important for the development of galls may be identified and provide potential clues as to the signalling mechanisms used in gall formation.

We decided to embark upon a small-scale "proteomics" approach comparing gall and non-gall protein signatures using SDS-PAGE gel electrophoresis, staining with Coomassie Brilliant Blue and using western blotting analysis. Although protein separation in one dimension according to molecular mass by SDS-PAGE does not identify a protein as

accurately as 2-D gels, it does yield meaningful information as separated protein bands can be isolated for partial amino acid sequence determination and this information used to identify the protein by comparison to the databases and by western blotting using specific antibodies where available.

In this chapter, I report work done on a variety of cynipid oak and rose galls at various stages of development to analyse inner-gall tissue protein signatures compared to non-gall tissue. Such protein signatures can also be used as molecular markers for gall formation and in chapter 6 I describe the development of a bioassay to detect the cynipid signalling molecules which induce expression of such proteins.

Results

4.2 Selection of inner-gall tissue for analysis of protein signatures

The inner-gall tissue is common to all cynipids and, as discussed in chapter 1, the transformation of the nutritive parenchyma into the nutritive cells occurs throughout development until all the tissue has been grazed and the larva is ready to pupate. The transformation of the parenchyma into the nutritive cells is controlled by the larva, as, if the larva is killed, the transformation ceases. It is likely, therefore, that expression of specific proteins within the nutritive cells are controlled by the larva. This intimate interaction between larva and inner-gall tissue provides an ideal system to analyse the qualitative and quantitative protein content of inner-gall tissue from different cynipid species. This work was carried out in collaboration with, and principally by, Dr Karsten Schönrogge, a postdoc in the laboratory and was published in *Plant Cell and Environment*

(Schönrogge, Harper and Lichtenstein., 2000, see appendix).

Initially we analysed two galls, an oak gall and a rose gall. For the oak gall we chose *Andricus quercuscalicis*, the "knopper gall", a single-chambered gall formed in the developing acorn by the agamic generation (collected from local English woodland). For the rose gall we chose that formed by the Canadian single-generation cynipid *Diplolepis spinosa* which forms multi-chambered stem galls in the meadow rose, *Rosa blanda*. Material for this was kindly provided by Professor Joe Shorthouse, Laurentian University, Ontario, Canada as part of a collaboration. Inner-gall tissue was dissected to remove the larval material and the cortex of the gall, and protein extractions of the inner-gall tissue were made (see section 2.3.1) and quantified using the Bradford assay (section 2.3.2). We also harvested and carried out protein extraction on leaf, stem and seed material from both oak and *Rosa rugosa* (*Rosa blanda* was not available) for comparison. Inner-gall protein extracts and non-gall tissue extracts were run on a gradient SDS-PAGE gel (6.5% - 20%) and visualised by staining with Coomassie Brilliant Blue.

Protein signatures from just the oak and a rose gall inner gall tissue are shown in Figure 4.1. Amongst the numerous protein bands visible for each gall, a 62kDA and a 42Kda band (see arrows in Figure) appears common to both. These bands, plus those of a number of other abundant proteins, were excised and used for partial amino acid sequence determination by Bryan Dunbar in a service provided by the Protein Facility at the University of Aberdeen. Many, however, were N-terminally blocked and could not be sequenced. Fortunately, the two proteins at 42kDa and 62kDa, abundant in both

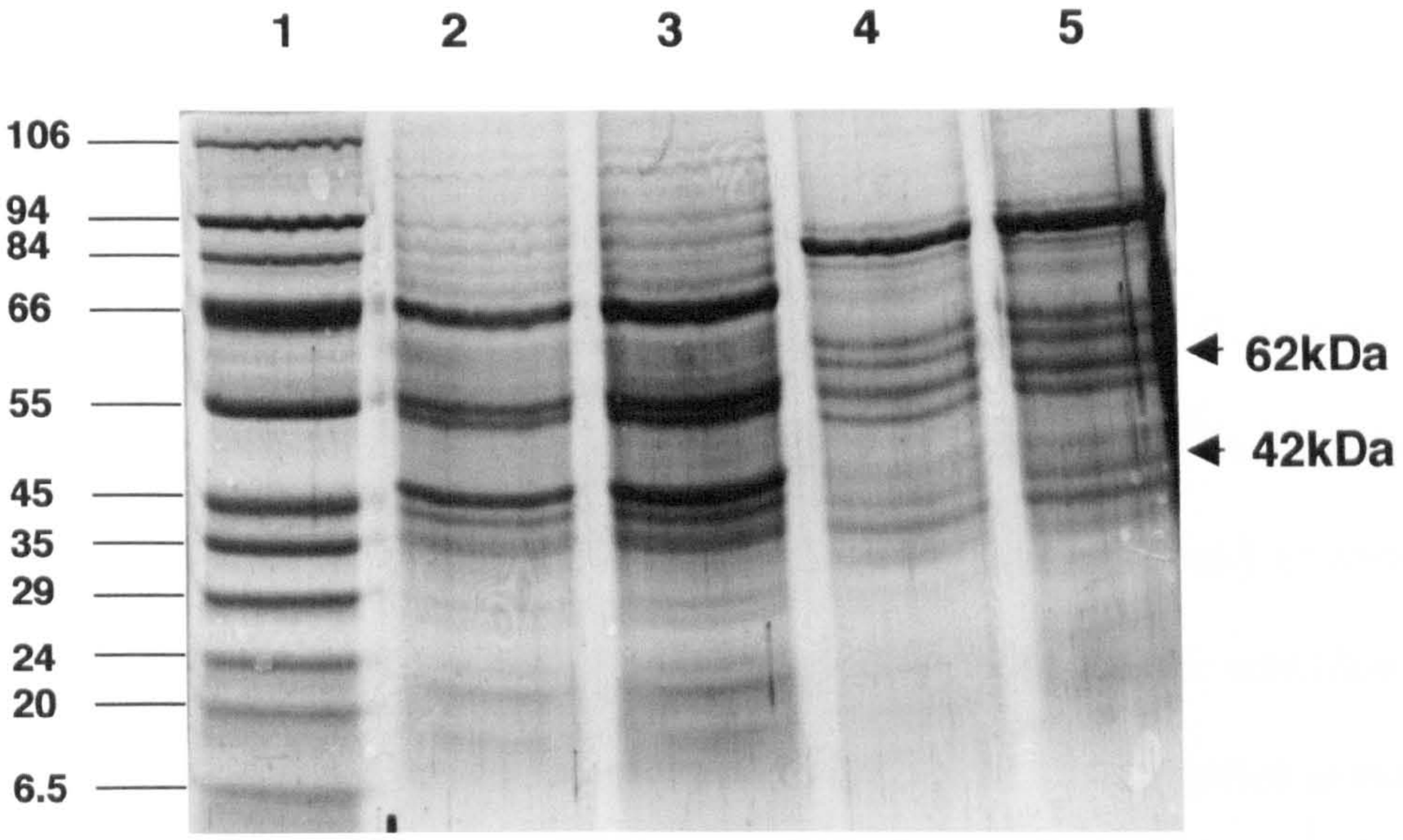


Figure 4.1 A gradient SDS PAGE gel (6.5%-20%) showing the protein signatures of oak and rose inner-gall tissue. Lane 1 molecular weight marker. Lane 2 *A.quercuscalicis* inner-gall signature 5µl. Lane 3 *A.quercuscalicis* inner-gall signature 10µl. Lane 4 *D.spinosa* inner-gall signature 5µl. Lane 5 *D.spinosa* inner-gall signature 10µl.

A

```
Aqc    L H A S P G S K K I V G V F Y
       : . : : : :   : : : : : : : :
FDH    L Q A S P G P K K I V G V F Y
```

B

```
Ds     D E AD X K E F V L T X D
             :   : : : : : : :
PDI    S S T D A K E F V L T L D
```

Figure 4.2 The sequence alignment of A) 43kDa *A.quercuscalicis* (Aqc) inner-gall protein to FDH from *S.tuberosum* and B) 62kDa *D.spinosa* (Ds) inner-gall protein to PDI from *M.sativa*.

A.quercuscalicis inner-gall tissue and *D.spinosa* inner-gall tissue, were successfully N-terminally sequenced.

4.2.1 Protein disulphide isomerase expression in gall tissue

An 11 amino acid N-terminal sequence of the 62kDa protein from *D.spinosa* was obtained and used for sequence comparison to amino acid databases. A 72.7% identity was found to protein disulphide isomerase (PDI) from Alfalfa (*Medicago sativa*), as shown in Figure 4.2.A (Shorrosh and Dixon, 1992). PDI is found in the endoplasmic reticulum and is involved in the refolding of proteins (Shimoni *et al.*, 1995). Antibodies to these proteins were obtained from Dr R.A. Dixon from The Samuel Roberts Noble Foundation Inc. Ardmore Oklahoma, USA, and used to confirm the identity of the inner-gall protein by western blotting. Five different inner-gall tissues were used for western analysis, the original two plus two additional oak and one additional rose gall. Inner-gall samples and non-gall tissues were run on a gradient SDS-PAGE gel (6.5% - 20%) and blotted onto Hybond C membrane using a semi-dry electroblotter. The membrane was blocked and incubated with the anti-PDI antibody, washed and incubated with the secondary antibody, and detected using ECL (section 2.3.4). Figure 4.3 shows the western blot using the PDI antibody. PDI is present in all the inner-gall tissues tested and was also detected in non-gall tissues leaf, stem and seed. This confirms the identity of the 62kDa protein as PDI and shows it is expressed throughout all the inner-gall. But as we are searching for inner-gall specific proteins which may give us an indication as to how gall formation is controlled and could possibly be used as a marker in the bioassay, PDI proved not to be of significant interest here as it is also in all non-gall tissues.

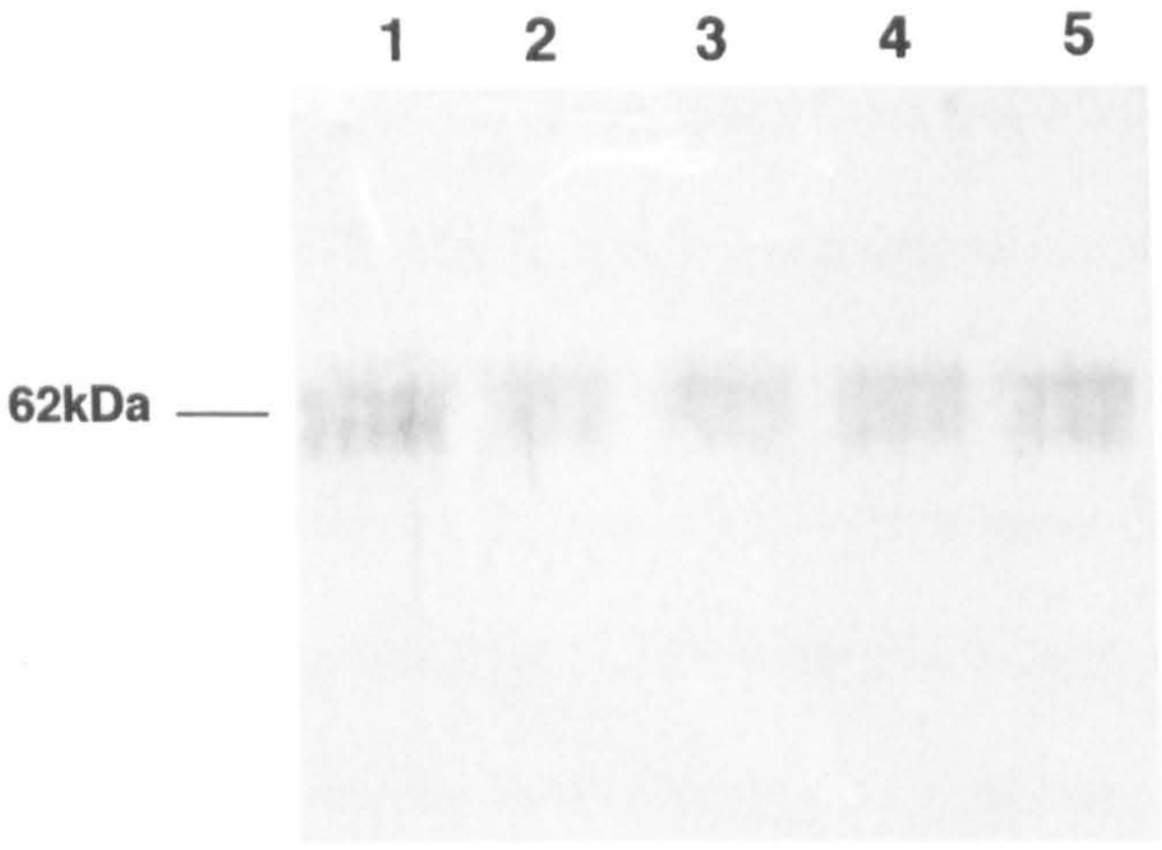


Figure 4.3. A western blot showing the hybridisation of PDI antibody to inner-gall tissue. Lane 1 *A.quercuscalicis* inner-gall tissue. Lane 2 *A.fecundator* inner-gall tissue.Lane 3 *A.kolleri* inner-gall tissue. Lane 4 *D.rosae* inner-gall tissue. Lane 5 *D.spinosa* inner-gall tissue.

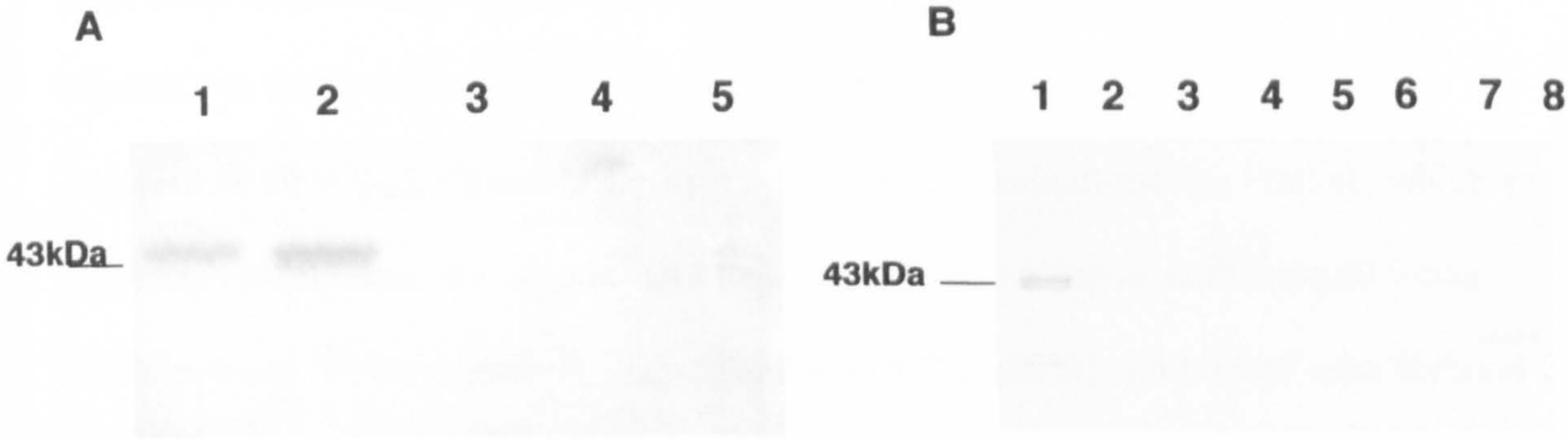


Figure 4.4 A western blot showing the detection of FDH in A) inner-gall tissue and B) non-gall tissue protein extracts. In A) FDH can be detected in 2 inner-gall tissues Lane 1 *A.quercuscalicis* inner-gall tissue. Lane 2 *A.fecundator* inner-gall tissue. Lane 3 *A.kolleri* inner-gall tissue. Lane 4 *D.rosae* inner-gall tissue. Lane 5 *D.spinosa* inner-gall tissue. In B) FDH can only be detected in *A.quercuscalicis* inner-gall tissue. Lane 1 *A.quercuscalici sinner*-gall tissue. Lane 2 leaf tissue. Lane 3 Stem tissue. Lane 4 Seed tissue. Lane 5 *D.rosae* inner-gall tissue. Lane 6 Leaf tissue. Lane 7 Stem tissue. Lane 8 Seed tissue.

4.2.2 Formate dehydrogenase expression in gall tissue

The 15 amino acid N-terminal sequence from the 43kDa *A.quercuscalicis* inner-gall protein shows a 86.7% identity to formate dehydrogenase (FDH) from potato (*Solanum tuberosum)*, as shown in Figure 4.2.B. FDHs are present in plants, mammals, bacteria, yeast and fungi, however, different forms exist depending on their location within the cell, cofactors and substrates. The FDH found to be similar to the inner-gall protein is a nuclear encoded, NAD-dependent FDH found in the mitochondria (Hourton-Cabassa *et al.*, 1998). Expression studies have been carried out on NAD-dependent FDH and it has been found to be highly expressed in non-photosynthetic tissues such as the potato tuber and expressed at low levels in leaves (Hourton-Cabassa *et al.*, 1998). It decomposes formic acid into hydrogen and carbon dioxide and is highly expressed when the cell is in stress, such as in dark or hypoxic conditions (Hourton-Cabassa *et al.*, 1998). An antibody against this NAD-dependent FDH in potato was obtained from Dr R Remy from UniversitÈ Paris Sud, Centre D'Orsay, Institute de Biotechnologe des Plantes, which was used for western analysis of inner-gall tissue. Inner-gall samples and non-gall tissue samples were run on a gradient SDS-PAGE gel (6.5% - 20%) and blotted onto Hybond C membrane using a semi-dry electroblotter. The membrane was blocked and incubated with anti-FDH antibody, washed and incubated with the secondary antibody, and detected using ECL (section 2.3.4). Western blotting confirmed the identity of the inner-gall protein as FDH, as shown in Figure 4.4.A. FDH is present in only two of the five types of galls tested, *A.quercuscalicis* and *A.fecundator*. It is not detected in any non-gall tissue, see Figure.4.4.B, suggesting that some gall tissues are under stress, whereas others are either not stressed or have overcome it. This may be achieved by the formation of

enlarged polytene inner-gall cells, which will be discussed further in chapter 5.

4.2.3 Putative biotin carboxylase carrier protein

Regardless of the antibody used in the western analysis of the gall tissue, when the western was carried out using streptavidin horseradish-peroxidase as the secondary antibody, a 35kDa band was routinely detected. The western method involved using a primary antibody which would bind to the specific protein and then a streptavidin peroxidase enhancer step which would bind to the primary antibody and provide peroxidase for the ECL reagents to react with. When a control western using no primary antibody was carried out, despite using stringent washes, the 35kDa band appeared in all the inner-gall tissues tested as shown in Figure 4.5.A., unexpectedly demonstrating that a streptavidin-binding protein is present in inner-gall tissue. When tested on non-gall tissues, the 35kDa band was only detected in seeds, as shown in Figure 4.5.B. The well documented and widely used streptavidin-biotin binding, suggests the protein may be naturally biotinylated, which would explain the detection when only streptavidin peroxidase is being used. Other non-biotinylated, streptavidin-binding proteins may exist, however, it is unlikely that their affinity to streptavidin is as great as biotin, and they would probably be removed during the washing process, or appear as faint non-specific background on the blot. The band being detected is very intense, even after extremely short exposures to the blot, and is therefore likely to be specific and explained by a biotin moiety attached to the protein.

There are six known biotinylated plant polypeptides ranging from 210kDa – 30kDa, involved in catalysing the reactions entailing the transfer of carboxyl groups and incorporation of CO_2 (Wurtele and Nikolau, 1990). The expression of these occurs mainly

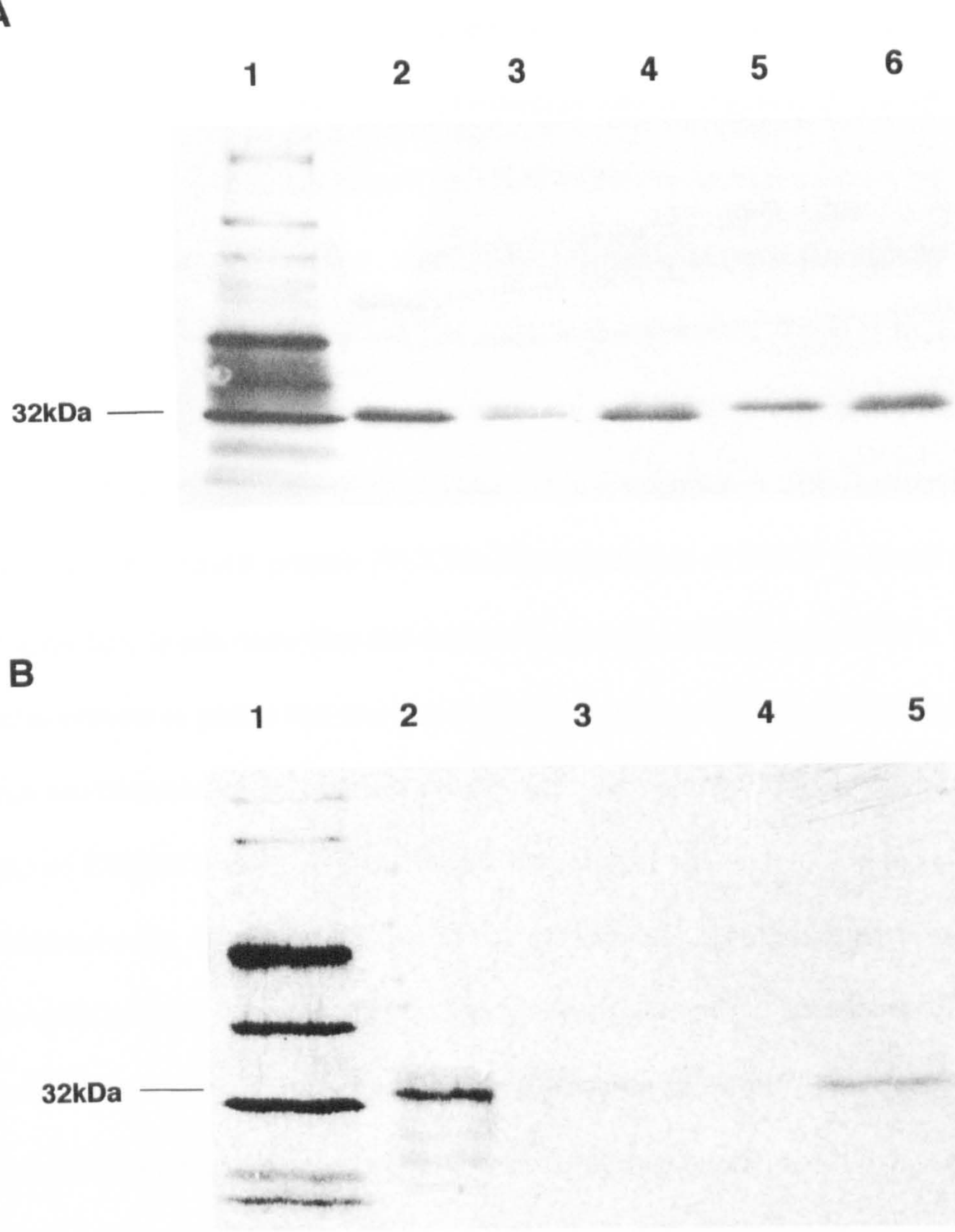


Figure 4.5 shows the detection of a biotinylated protein in A) Inner-gall tissue and B) Non-gall tissue protein extracts. In A) the biotinylated protein can be detected in all the inner-gall tissues. Lane 1 shows the biotinylated molecular weight marker. Lane 2 *A.quercuscalicis* inner-gall tissue. Lane 3 *A.fecundator* inner-gall tissue. Lane 4 *A.Kolleri* inner-gall tissue. Lane 5 *D.rosae* inner-gall tissue. Lane 6 *D.spinosa* inner-gall tissue.
In B) detection can only be seen in the seed protein extract. Lane 1 shows the biotinylated molecular weight marker. Lane 2 shows *A.quercuscalicis* inner-gall tissue. Lane 3 Leaf tissue. Lane 4 Stem tissue. Lane 5 Seed tissue showing expression of biotinylated protein.

in seeds, which corresponds to our western results, detecting the 35kDa band in seeds and inner-gall. The actual identification of the inner-gall tissue protein can be speculated from the identification of a biotinylated protein in oil seed rape, as biotin carboxylase carrier protein (BCCP) (Elborough,1996). The protein runs at 35kDa and is a subunit of a class II acetyl CoA-carboxylase (ACCase), involved in the production of triacylglycerol lipids, a rich source of energy. The size, biotinylated characteristic and the expression in seeds, all link BCCP to our inner-gall protein detected in the westerns. Type II ACCase is an essential enzyme in the synthesis of fatty acids, and is found in plants and bacteria and is made up of a dimer of biotin carboxylase (BC), a tetramer of carboxyltransferase (CT) and biotin carboxyl carrier protein (BCCP). The expression of BCCP is found mainly in seeds, however low levels were detected in roots and leaves (Elborough, 1996). Type I ACCase is also present in plants and can also be found in mammals and yeast. This consists of a large multifunctional polypeptide made from two identical subunits with the molecular mass of 220kDa and BC, CT and BCCP domains. It can be found in the cytosol of epidermal cells, where as the type II ACCase is found in the chlorplasts and plastids of mesophyl cells (Elborough, 1996). The expression of BCCP belonging to type II ACCase, was investigated by Elborough (1996) and found to be nuclear-encoded, localised to the choroplast, with a peak in expression at mid embryo development, when the lipid synthesis was at a maximum. After this the expression decreased gradually until desiccation. They also found differential expression of additional biotinylated proteins and protein of 50kDa, 70kDa, 75kDa and 88kDa could also be detected in the embryo at different stages of development, although, functional analysis was not carried out on the additional biotinylated proteins within this study.

Other studies have shown the expression pattern of the biotinylated proteins in the seeds of pea (*Pisium sativum*) and soybean (Duvel *et al.*, 1994, 1995; Neto *et al.*, 1997). In soybean, three groups were investigated to determine if there is a relationship between their expression and development of seed desiccation tolerance. The first group is a set of three bands with an average weight of 85kDa, the second a 75kDa single protein and the third a group of 3 bands with a mean weight of 35kDa. The study of these 3 groups of proteins in seeds provided a clear pattern of their detection using the biotin-streptavidin detection system. The 85kDa and 75kDa groups are not expressed or are at low concentrations in the early stages of seed development but accumulate in the cotyledons and the embryo axis as the seed matures. The 35kDa group are expressed mainly in the developing embryo and as the seed germinates the concentration decreases. In pea, 3 groups of biotinylated proteins are detected in mature dried seed, 65kDa, 75kDa and 200kDa. The 75kDa and 200kDa proteins were found to be involved in acetyl-Co enzyme carboxylase and 3-methylcrotonyl-CoA carboxylase activity respectively. The 65kDa protein, however, does not show any of the biotin-dependent carboxylase activity. It is specifically expressed in seeds in the cotyledons and the embryonic axis and is expressed late in maturation just before desiccation. Interestingly, the 35kDa protein is not detected in peas, which could be explained by the low oil content of the seed, 2% of the mass compared to 45% of the mass in rape (Elborough 1996). The expression of this BCCP in the inner-gall tissue would be plausible as the nutritive cells are also high in lipids, used as nutrients for the larva.

4. 3 Analysis of protein signatures and putative BCCP expression in other species of gall

To investigate these findings further (Schönrogge, Harper and Lichtenstein, 2000), I decided to examine the protein signatures in different galls at different stages of their development and look for the presence of the biotinylated protein. I also wanted to determine if any of the other groups of biotinylated proteins are expressed in early or late stages of gall formation. To achieve this I carried out protein extraction and quantification of the inner-gall tissue using several cynipid gall species. SDS-PAGE analysis and western blotting using the biotin-streptavidin detection system enabled the biotinylated proteins to be identified and expression patterns established.

The tissue distribution of the proteins within the galls is also of interest, indicating where the lipid production is concentrated and if the distribution of the protein changes throughout development. The analysis of putative BCCP distribution will be presented and discussed in chapter 5.

4.3.1 Protein signatures and expression of biotinylated proteins

Protein extractions of the dissected inner-gall tissue were made (see section 2.3.1) and quantified using the Bradford assay (section 2.3.2). 20µg of inner-gall protein extracts from different gall species at different stages of development were run on a gradient SDS-PAGE gel (6.5% - 20%) and visualised by staining with Coomassie Brilliant Blue, which enables predominant differences in band patterns to be visualised.

To determine if the biotinylated protein is differentially expressed throughout

development western blotting was used. 10µg of the inner-gall samples from different developmental stages were run on a gradient SDS-PAGE gel (6.5% - 20%) and blotted onto Hybond C membrane using a semi-dry electroblotter. The membrane was blocked and incubated with streptavidin-horseradish peroxidase and detected using ECL (section 2.3.4.3).

To ensure this detection method was efficient, pea seed extract was first used in western analysis to determine if all the biotinylated proteins could be observed. The pea embryo and cotyledon were separated and protein extracts were run on a SDS-PAGE gradient gel, blotted and incubated with streptavidin-horseradish peroxidase. Figure 4.6 shows the detection of four biotinylated proteins at 85kDa, 75kDa, 65kDa and 35kDa. The desiccated mature seed shows the expression of all four biotinylated proteins, although the 200kDa protein reported in mature desiccated seeds by Duval *et al.* (1995) is not detected. Also the 85kDa and the 35kDa proteins detected here, were not detected in the extracts tested by Duval *et al* (1995) on peas, but were found in soybean (Neto *et al.*, 1997). This could be due to different extraction methods, or the developmental stage of the sample. Here, the embryo extract at 24 and 48 hours after imbibition shows the 35kDa and 85kDa protein. In the cotyledon, 24 hours after imbibition the 35kDa, 65kDa and 85kDa proteins are clearly detected, although the 75kDa can be seen as a faint band. The 85kDa band detected here is seen as a single band and not as a triplet, similarly the 35kDa band is not seen as a triplet of bands, as reported in soybean by Neto *et al.* (1997), only as a doublet. This suggests that the separation of proteins using the minigel system is not as efficient as the method used by Neto *et al.* (1997), although the method is able to specifically detect biotinylated proteins and, therefore, will detect the presence of biotinylated proteins in gall

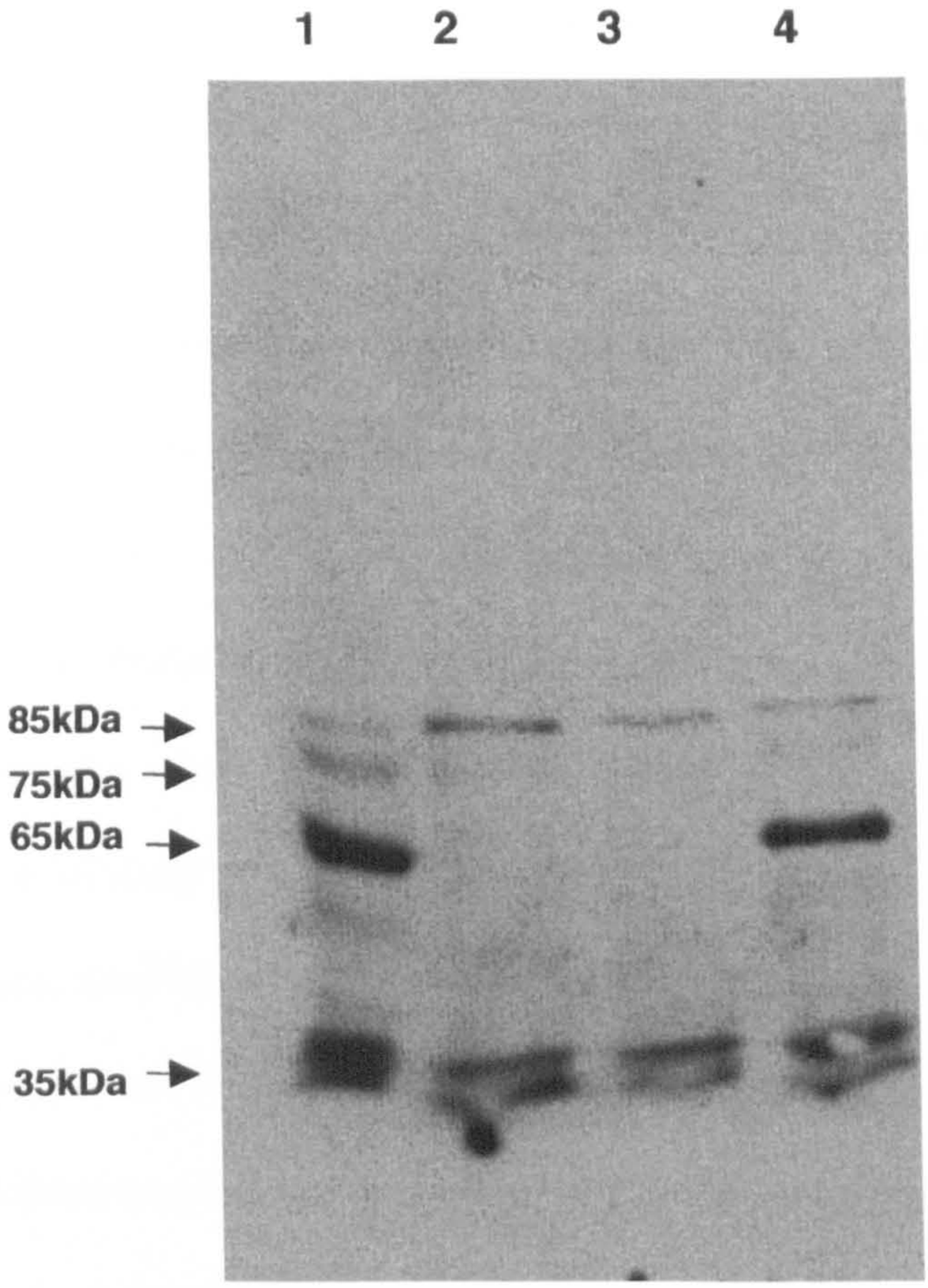


Figure 4.6 shows the detection of biotinylatd proteins in pea embryoand cotyledon protein extract. Lane 1 contains the desiccated seed protein extract. Lane 2 contains embryo extract 24 hours after imbibition. Lane 3 contains embryo protein extract 48 hours after imbibition. Lane 4 contains cotyledon extract 24 hours after imbibition.

tissue, if present at sufficient concentrations.

4.3.2 Gall collections and dissection of inner-gall tissue

The galls chosen for analysis were the oak galls: *Biorhiza pallida, Neuroreus quercusbaccarum, Cynips quercusfolli Andricus quercuscalicis, Andricus fecundator.* These were chosen as they were known to be induced in areas in and around London, guaranteeing regular collections and reducing the time between sample collection and processing. Collections were made once or twice weekly between the end of April to June for the spring generation and July to August for the autumn generation. The galls were separated into stages of development and then either dissected immediately into inner-gall tissue and larva, or snap frozen in liquid nitrogen and stored at –70°C. Cynipids do not moult, therefore, developmental stages were defined by the width of the larval head capsule, the length and width of the larva and the length and width of the larval chamber. Distinct gall characteristics, such as the presence of the egg, thickness of inner-gall tissue and presence of sclerenchyma capsule were used together with the larval statistics to divide the larvae into four stages of development. As each gall is species specific, pattern and the size of larva and chamber differ between species, therefore, each gall has individually defined stages of development. The protein signatures of inner-gall tissue, at different stages of development, were analysed using Coomassie Brilliant Blue staining and detection of the putative BCCP was carried out using streptavidin-horseradish peroxidase, both of which are discussed below. To introduce each gall, the life cycle and the defined stages of development will first be discussed.

4.3.2.1 Protein signatures of *Biorhiza pallida* throughout development

Biorhiza pallida, the "oak apple", has two generations a year forming on *Q.robur*. The unisexual generation forms multilocular galls on the host roots, which take two years to develop. The adults emerge at the end of winter and lay eggs in buds. The bisexual generation forms in spring as a multilocular bud gall on *Q.robur*. The adults emerge June-July and the cycle begins again. I chose the bud gall due to its abundance and accessibility. The stages of development are defined in the table below and schematic diagrams of the gall chamber throughout development are summarised in Figure 4.7.A. External and internal morphology of the whole gall can be seen in chapter 5.

Table 4.1 The defined stages of development for *B.pallida*

Stage of development	Chamber size		Larva size		
	Length (mm)	Width (mm)	Length (μm)	Width (μm)	Head capsule diameter (μm)
Stage 1	1 - 1.25	0.5 – 0.75	306 –371	274 – 323	160 - 306
Stage 2	1.25 - 2.25	0.625 - 1.25	387 – 790	339 –548	323 - 403
Stage 3	2.375 - 3.125	1.25 - 2.125	790 - 1290	565 – 807	419 - 516
Stage 4	3.125 - 3.5	1.875 - 2.125	1300 - 1610	807 – 1250	532 - 645

Figure 4.8.A shows inner-gall protein extracts of *B.pallida* at four stages of development, the arrows to the right of the gel indicate the molecular mass and lanes in which the different bands are present. The protein extracts do not appear to vary considerably between stages. There appear to be differences in band intensities and additional bands in some stages. A summary of the *B.pallida* protein signature compared to the other signatures analysed is shown in Table 4.2.

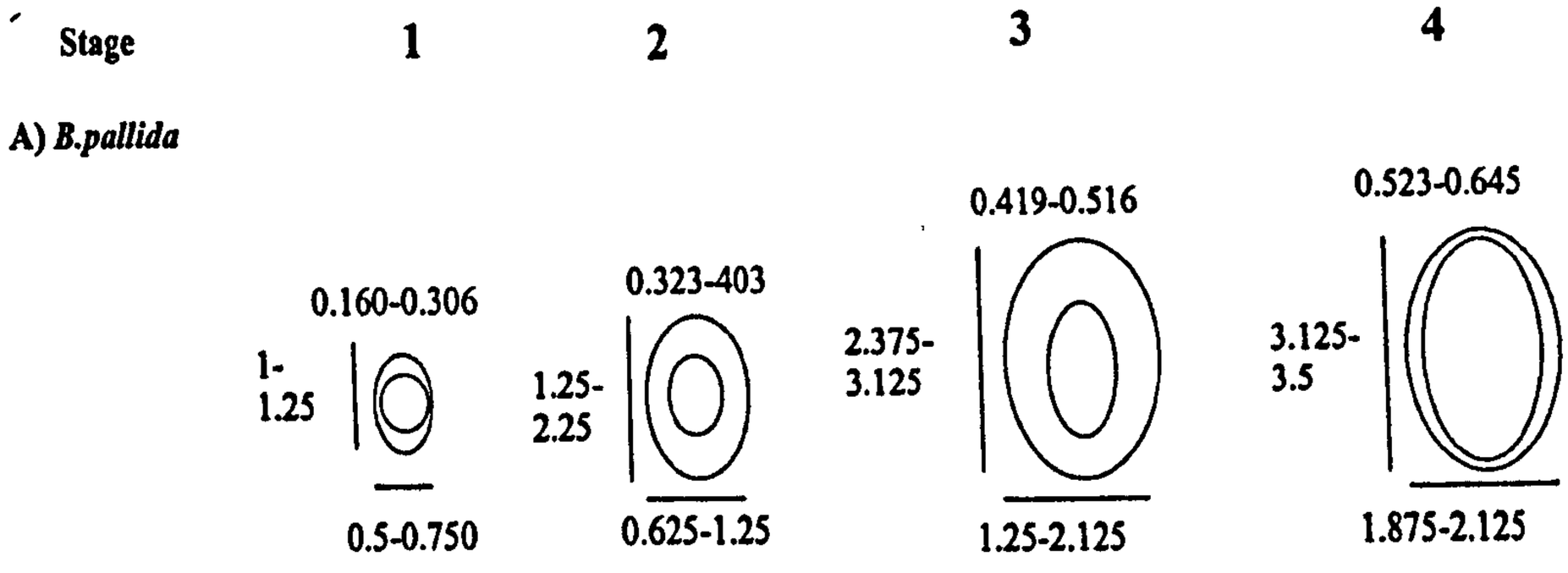


Figure 4.7 The chamber sizes for the defined stages of development showing the length and width of the chamber and the larval head capsule diameter (above the chamber) in mm. (A) *B.pallida.* (B) *N.quercusbaccarum* (C) *C.quercusfolii.* (D) *A.quercuscalicis.* (E) *A.fecundator.*

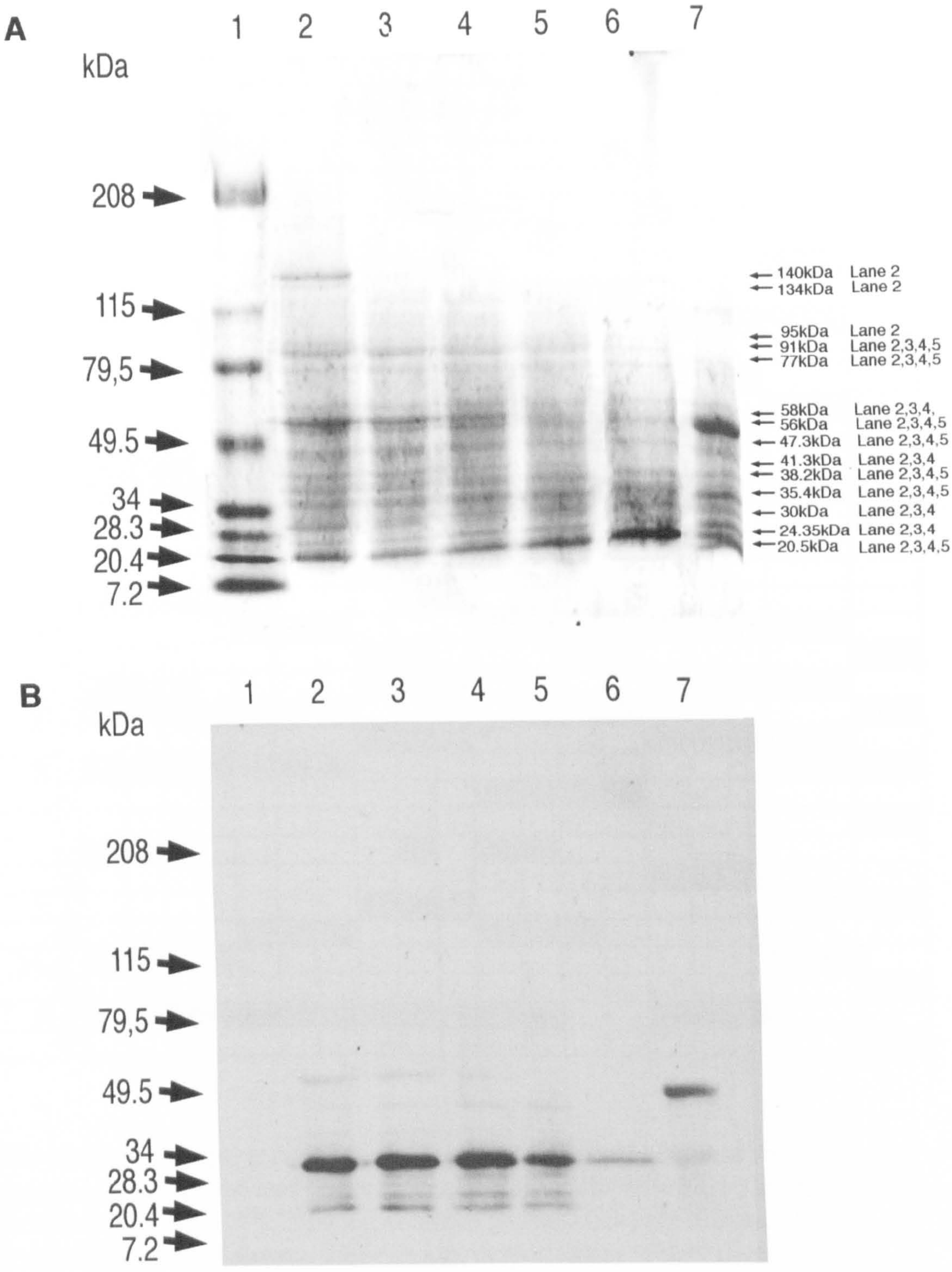


Figure 4.8 (A) SDS-PAGE gradient gel (6.5%-20%) and (B) western blot showing protein extracts from *B.pallida* inner-gall tissue at 4 stages of development. In (B) the extracts were probed with streptavidin-horseradish peroxidase to analyse expression of biotinylated proteins. Lane1 contains the wide range molecular weight marker. Lane 2 20µg stage 1 *B.pallida* inner-gall protein extract. Lane 3 20µg stage 2 *B.pallida* inner-gall protein extract. Lane 4 20µg stage 3 *B.pallida* inner-gall protein extract. Lane 5 20µg stage 4 *B.pallida* inner-gall protein extract. Lane 6 acorn protein extract. Lane 7 oak leaf protein extract.

Size kDa	*B.pallida*				*Nqb*			*Cqf*			*Aqc*				*Af*			Acorn	Leaf
	1	2	3	4	1	2	3	1	2	3	1	2	3	4	1	2	3		
140	■																		
134	■																		
115														■					
106																			■
95	■								■	■					■	■	■		
91	■	■	■	■															
88					■	■	■	■	■	■								■	
86											■	■	■	■					
77	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	
73.6										■									■
72															■	■	■		
64																		■	
60															■	■	■		
58	■	■	■			■	■	■	■	■									
56	■	■	■	■	■	■	■	■	■	■	■	■	■	■				■	
51																			■
49															■	■	■		
48.7								■	■	■									
47.3	■	■	■	■					■	■									
46.6					■	■	■				■	■	■	■	■	■	■	■	
43.5								■					■						■
41	■	■	■					■	■						■	■	■	■	
38.2	■	■	■	■	■	■	■						■	■					■
37								■	■	■					■	■	■	■	
35.4	■	■	■	■	■	■	■											■	■
34											■	■	■	■					
31																			■
30	■	■	■						■		■	■							
28															■	■	■		
27.3								■	■	■								■	■
26					■	■	■				■	■	■						
24.35	■	■	■	■														■	
22																			■
20.5	■	■	■	■	■	■	■	■	■	■	■	■	■			■	■		
17																			■

Table 4.2 Protein signatures of cynipid galls at different stages of development compared to acorn and leaf tissue extract. Black cells indicate the expression of a protein at stages 1,2,3 or 4, showing common proteins between different species of gall and acorn and leaf tissue. *Nqb = N.quercusbaccarum. Cqf = C.quercusfolii, Aqc = A.quercuscalicis, Af = A.fecundator*

Looking in more detail, at stage 1 there are bands at 140kDa,134kDa and 95kDa, not seen in the other 3. A 91kDa band is present in all 4 stages, although this seems to fade as development progresses. A 77kDa faint band is seen which decreases in intensity towards stage 4. There is a more intense band at 58kDa that also decreases in intensity as the gall develops and is not visible in stage 4. A slightly lower band at 56kDa is present at equal intensities in stages 1-3 and decreases in 4, but is still faintly visible. A 47.3kDa band appears at equal levels throughout and a 41kDa band is expressed in the first 3 stages but is not present in stage 4. A 38.2kDa and 35.4kDa appear at the same intensity in all 4 stages. A 30kDa band is present in stages 1, 2 and 3, and bands at 24.35kDa and 20.5kDa are present in all stages.

Expression of biotinylated proteins throughout *B.pallida* development is shown in Figure 4.8.B. All the stages express the 35kDa biotinylated protein and the latest stage appears to express this at lower levels. The slight decrease in expression in the final stage may be explained by the reduced nutritive requirements of the mature larva. The remaining nutritive cells, therefore, reduce production of nutrients including lipids. The Coomassie stained gel does show a band of similar molecular mass at 35kDa, which could be the biotinylated protein, expressed at low levels in all stages. Western blotting is more sensitive than Coomassie staining, explaining the intense signal detected in the western compared to the relatively low concentration of the protein stained with Coomassie Brilliant Blue. The 35kDa protein is not one of the more abundant proteins in the total protein signature, suggesting the hybridisation by streptavidin is specific and is not non-specific binding to a highly abundant protein.

The acorn protein extract, as the positive control, is expressing the same size biotinylated protein as the inner-gall extracts. Interestingly, the levels of expression of the putative BBCP in the inner-gall tissue are up-regulated at all stages compared to the level of expression detected in the acorn, suggesting higher lipid production in the inner-gall. The inner-gall extracts contain faint bands below the intense signal, which is not seen in the acorn extract. These fainter bands could be degradation products of the clearly abundant 35kDa protein. The biotinylated protein is less abundant in the acorn, and any degradation products would probably be at undetectable concentrations.

Faint bands can be seen at 55kDa and 42kDa in the inner-gall samples, although, these are barely detectable and probably non-specific signal. No additional biotinylated proteins were detected in either the inner-gall tissue or acorn protein extract. Acorns of different stages of development were used, however, only the 35kDa protein is ever detected. The other biotinylated proteins, discussed earlier, may be present at low concentrations but are not detected using our detection method. Further analysis using higher concentrations of protein and specific antibodies may determine this, however, for this investigation it is not necessary. The leaf extract should contain no, or very low levels of the 35kDa protein, and as expected, only a faint 35kDa band is detected in the leaf extract, however, a higher band at 50kDa is being detected. This could be one of the other biotinylated proteins, although one of this size has not been identified, or possibly non-specific binding to the abundant 50kDa protein. This can clearly be seen on the coomassie stained gel and is probably the large subunit of RUBISCO (ribulose bisphosphate carboxylase).

4.3.2.2 Protein signatures of *Neuroterus quercusbaccarum* throughout development

Like *B.pallida, N.quercusbaccarum* has two generations, a spring and an autumn gall. The unisexual autumn gall forms as small discs (4-6mm) on the underside of *Q.robur* leaves. At maturity these fall from the leaves and over-winter in the leaf litter, with adults emerging in April. They oviposit their eggs and the spring bisexual generation forms as a monolocular, green, spherical gall (5-7mm) on the axis of the catkins or on the underside of the leaves. The galls available from our chosen field sites were the bisexual generation leaf galls. The stages of development are defined in the table below and schematic diagrams of chambers are summarised in Figure 4.7.B. External and internal morphologies of the whole gall can be seen in chapter 5.

Table 4.3 The defined stages of development for *N.quercusbaccarum*

Stage of development	Chamber size		Larva size		
	Length (mm)	Width (mm)	Length (µm)	Width (µm)	Head capsule diameter (µm)
Stage 1	1.125-1.375	1.125 - 1.375	645 - 807	161 - 403	161 - 323
Stage 2	1.5 - 2	1.5 - 2	807 – 1500	403 - 871	323 - 726
Stage 3	2.625 - 3.5	2.625 - 3.25	1516 - 2613	887 – 1370	726 - 887

The protein signatures of *N.quercusbaccarum* at three stages of development can be seen in Figure 4.9.A lanes 1,2 and 3. The stages show similar signatures to each other

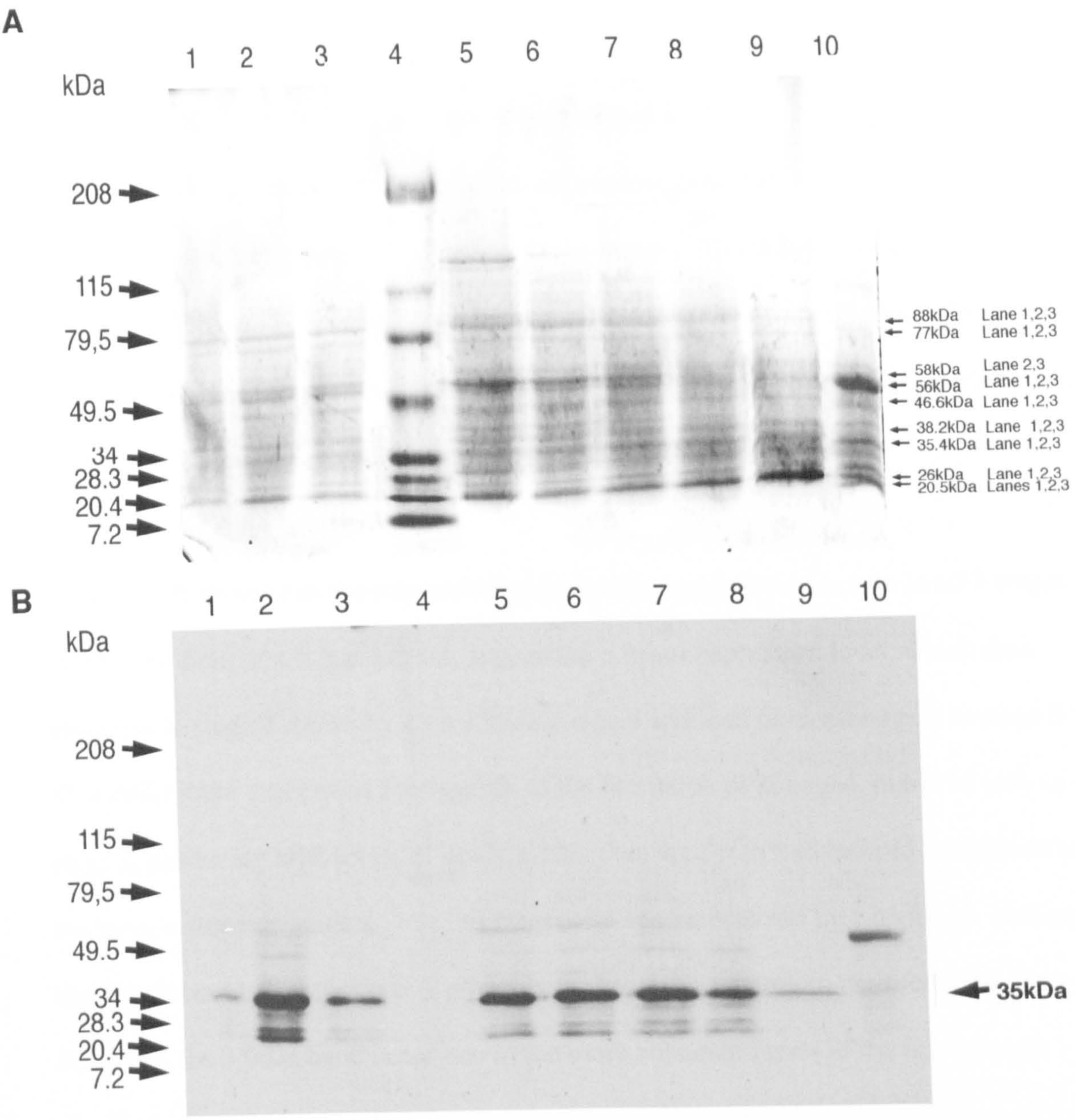


Figure 4.9 (A) SDS-PAGE gradient gel (6.5%-20%) and (B) western blot showing protein extracts from *N.quercusbaccarum* inner-gall tissue at 3 stages of development. In (B) the extracts were probed with streptavidin-horseradish peroxidase to analyse expression of biotinylated proteins. Lane 1 20µg stage 1 *N.quercusbaccarum* inner-gall protein extract. Lane 2 20µg stage 2 *N.quercusbaccarum* inner-gall protein extract. Lane 3 20µg stage 3 *N.quercusbaccarum* inner-gall protein extract. Lane 4 contains the wide range molecular weight marker. Lane 9 acorn protein extract. Lane 10 oak leaf protein extract.

with one or two bands missing in the stage 1 and 3. The protein signatures are summarised in Table 4.2. The highest band at 88kDa is present in all 3 stages and a 77kDa band is also present in all stages but decreases as development progresses. The 58kDa and 56kDa bands seen in *B.pallida* are present, however, the 58kDa band is not visible in stage1 but is present in stages 2 and 3 and the 56kDa band is expressed in all stages. 46.6kDa and 38.2kDa bands are present in all stages as are 35.4kDa, 26kDa and 20.5kDa bands. The 35.4kDa, 38.2kDa and 20.5kDa bands are also seen in *B.pallida* and the 47.3kDa band in *B.pallida* is very close to the 46.6kDa present here.

Figure 4.9.B shows that the expression of the biotinylated protein occurs in all 3 stages. In stage 1 the detection is less intense, suggesting a lower expression level, which then increases in stage 2 shown by a very intense signal and then decreases again in stage 3. This differential expression corresponds to the formation of enlarged, nutritive cells in stage 2, producing high levels of lipids, which then appear to reduce lipid production as the larva matures in stage 3. The 35kDa protein can be detected by Coomassie staining and is present in all stages and is probably the biotinylated protein detected here. As in *B.pallida*, the 35kDa band is not one of the more abundant bands of the total protein signature, suggesting that the western signal is specific. There are large amounts of probable degradation products in the inner-gall samples, also seen in *B.pallida*. Faint bands at 55kDa and 41kDa detected in *B.pallida* are also detected, which are likely to be non-specific signal due to the low intensity of the signal and there are no known biotinylated proteins of this size. The acorn shows the detection of a biotinylated protein, the same size as detected in the inner-gall extracts.

4.3.2.3 Protein signatures of *Cynips quercusfolli* throughout development

Cynips quercusfolii has two generations a year, the bisexual generation forms unnoticeable bud galls in spring. The unisexual generation, like *N.quercusbaccarum,* is a monolocular, pale green, spherical gall (15-25mm), forming on the underside of *Q.robur* leaves. This gall is larger than *N.quercuscalicis* and can be red in colour. The stages of development are defined in the table below and schematic diagrams of the chamber are summarised in Figure 4.7.C. External and internal morphologies of the whole gall can be seen in chapter 5.

Table 4.4 The defined stages of development for *C.quercusfolii*

Stage of development	Chamber size		Larva size		
	Length (mm)	Width (mm)	Length (mm)	Width (mm)	Head capsule diameter (μm)
Stage 1	1.5 -2	1.375 - 2	0.484 – 0.645	0.452 – 0.645	160 - 323
Stage 2	2.25 - 3.12	2.25 - 3.125	1.13 – 3.226	1.13 - 1.936	323 - 726
Stage 3	3.125 - 4.375	3.125 - 4.375	3.226 – 4.130	1.94 - 2.42	742 – 1370

Figure 4.10.A. shows the protein signatures throughout 3 stages in *C.quercusfolii.* Overall the signature is very similar to *N.quercusbaccarum*, which corresponds to their similar pattern of development. The expression is summarised with the other protein signatures in Table 4.2. The largest band at 95kDa is not present in stage 1 but is present in stage 2 and 3. A band at 88kDa increases with intensity as development progresses and a 77kDa band decreases in intensity towards stage 3, both of which are also seen in *N.quercusbaccarum.* A 73.6kDa band is present only in stage 3. The 58kDa

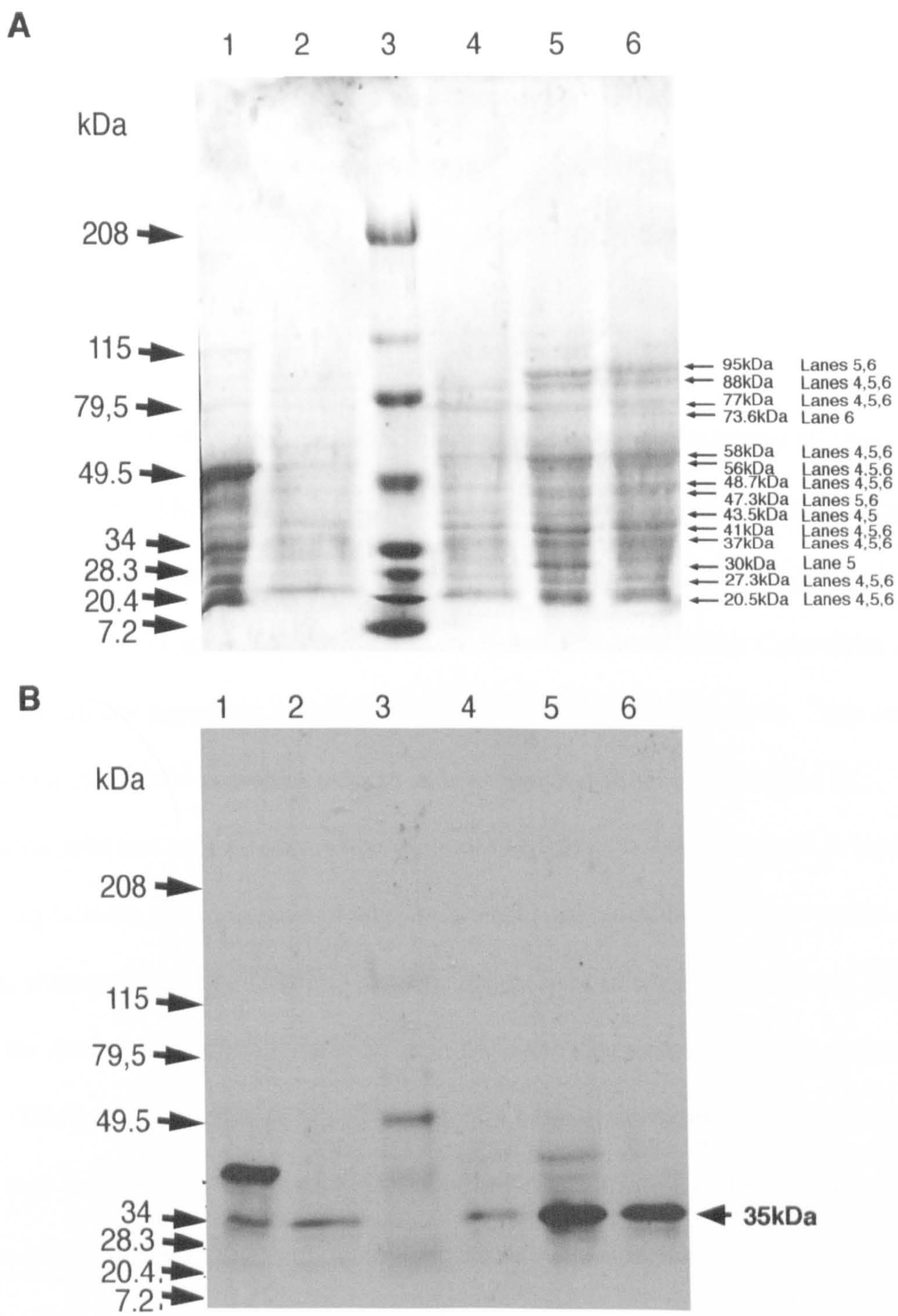


Figure 4.10 (A) SDS-PAGE gradient gel (6.5%-20%) and (B) western blot showing protein extracts from *C.quercusfolii* inner-gall tissue at 3 stages of development. In (B) the extracts were probed with streptavidin-horseradish peroxidase to analyse expression of biotinylated proteins. Lane1 oak leaf protein extract. Lane 2 acorn protein extract. Lane 3 contains the wide range molecular weight marker. Lane 4 20µg stage 1 *C.quercusfolii* inner-gall protein extract. Lane 5 20µg stage 2 *C.quercusfolii* inner-gall protein extract. Lane 6 20µg stage 3 *C.quercusfolii* inner-gall protein extract.

and 56kDa bands increase from stage 1 to 3, as in *N.quercusbaccarum*. The 48.7kDa, 41kDa, 37Kda, 27.3kDa and 20.5kDa bands are present in all stages at equal intensities. A 47.3kDa band is missing in stage 1 but present in stages 2 and 3. A 43.3kDa band is present in stages 1 and 2 but absent in stage 3. A 30kDa band is present only in stage 2.

Figure 4.10.B. shows the detection of the biotinylated protein throughout all the 3 stages in *C.quercusfolii*. The intensity of stage 2 is higher, which corresponds to the formation of large lipid filled cells in the gall. In stage 3 the intensity remains high, however, is slightly lower than stage 2. Interestingly there is no band detected by Coomassie at 35kDa in the total protein signature, despite a clear signal from western analysis. This would suggest that the 35kDa protein is present in low concentrations not detected by Coomassie, and can only be seen when the more sensitive western analysis is used. There are no degradation products seen in any of the inner-gall samples, as observed in other species, although there are faint non-specific bands seen in stage 2. The acorn extract shows the detection of a biotinylated protein the same size as that observed in the gall tissue. The leaf extract also shows detection of a band at the same molecular weight, which may be BCCP as this can be present at low concentrations. There is also an intense band at 42kDa in the leaf extract, which is probably non-specific as it is an abundant protein, as shown by the Coomassie stained gel and there are no known biotinylated proteins of this size. There is some non-specific binding on this blot as one of the molecular weight marker bands is being detected. Despite the non-specific binding, the specific signal is very intense and clearly distinguishable.

4.3.2.4 Protein signatures of *Andricus quercuscalicis* throughout development

A.quercuscalicis has two generations each year, one bisexual, monolocular spring gall formed on the catkins of *Quercus cerris* and a unisexual, autumn gall formed on acorns of *Quercus robur.* This monolocular acorn gall has spread to England from Europe and is quickly spreading across Britain and Ireland and due to its abundance was chosen as one of the gall species to analyse. The stages of development are defined in the table below and schematic diagrams of the chamber are summarised in Figure 4.7.D. External and internal morphologies of the whole gall can be seen in chapter 5.

Table 4.5 The defined stages of development for *A.quercuscalicis*

Stage of development	Chamber size		Larva size		
	Length (mm)	Width (mm)	Length (mm)	Width (μm)	Head capsule diameter (μm)
Stage 1	1.25 - 3.125	0.625 - 2.5	0.625 -1.25	0.5 - 1	160 - 323
Stage 2	2.625 - 3.375	2.2 - 3.25	1.372 - 1.75	1 - 1.25	323 - 484
Stage 3	3.5 - 3.875	3.375 - 3.75	1.875 - 2.5	1.25 - 1.165	484 - 597
Stage 4	4 - 4.25	3.875 - 4.125	2.625 - 3.125	1.625 - 1.875	597 - 726

The protein signatures of *A.quercuscalicis* can be seen in Figure 4.11.A. and are summarised in Table 4.2. These vary more than *N.quercusbaccarum* and *B.pallida* with predominant bands being 77kDa, 56kDa and 46.6kDa. There is a faint band, only clearly visible in stage 4, at 115kDa. The 77kDa band is not visible in stages 1 and only slightly in stage 2, whereas in stage 3 and 4 it is one of the more intense bands. The higher band of 86kDa is less intense but clearly visible in stages 3 and 4 and only just visible in 1 and 2. The 56kDa and 46.6kDa band increase in intensity throughout development. In stage 1

both are expressed at low levels and gradually increase in each stage. The band immediately under this at 43.5kDa is expressed at high levels in stages 3 and 4 but not in stages 1 and 2. Conversely the 34kDa, 30kDa and 20.5kDa bands are expressed at higher levels in stages 1 and 2 and are not visible in stage 3 and 4. A 26kDa band can be seen in stages 1, 2 and 3.

Figure 4.11.B shows the expression of the biotinylated protein in all four stages of development. Stage 1 and 2 show the most intense signals, which decrease in stage 3 and again to low levels in stage 4. The levels appear to be higher in stage 2 than stage 1 which corresponds to the formation of inner-gall tissue. In stage 1 there is no, or only a thin layer of inner-gall tissue and in stage 2, the inner-gall tissue forms around the egg-like-structure in which the larva is contained, and both are expressing the biotinylated protein. This will be discussed further in chapter 5. In stage 3 the signal decreases which could be explained by the regression of the egg, reducing the BCCP expressing cells and therefore concentration in the total protein extraction, also shown in chapter 5. A band of 35kDa can be detected by Coomassie staining and this is present at higher levels in stage 1and 2 and then disappears in stage 3 and 4. This corresponds to the western signal reducing in intensity in stages 3 and 4 showing that the 35kDa protein is still expressed in these stages, although can not be detected by Coomassie Brilliant Blue staining. There are some faint, non-specific bands in stage 2 and 4 and also in the marker lane, however, the true specific signals are clearly visible. The acorn sample shows the detection of the biotinylated protein at the same size as the inner-gall tissue.

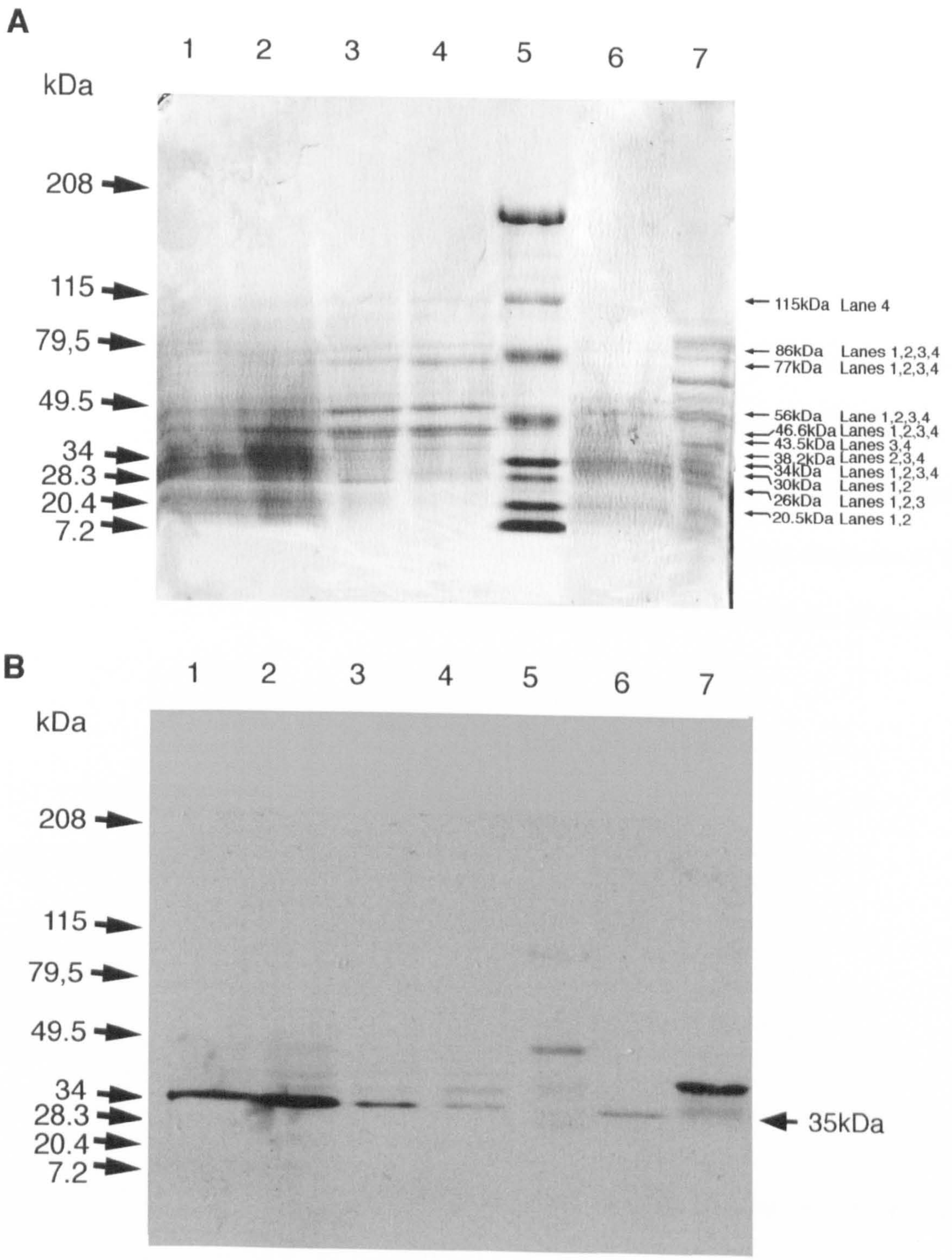


Figure 4.11 (A) SDS-PAGE gradient gel (6.5%-20%) and (B) western blot showing protein extracts from *A.quercuscalicis* inner-gall tissue at 4 stages of development. In (B) the extracts were probed with streptavidin-horseradish peroxidase to analyse expression of biotinylated proteins. Lane1 20µg stage 1 *A.quercuscalicis* inner-gall protein extract. Lane 2 20µg stage 2 *A.quercuscalicis* inner-gall protein extract. Lane 3 20µg stage 3 *A.quercuscalicis* inner-gall protein extract. Lane 4 20µg stage 4 *A.quercuscalicis* inner-gall protein extract. Lane 5 contains the wide range molecular weight marker. Lane 6 acorn protein extract. Lane 7 oak leaf protein extract.

4.3.2.5 Protein signatures of *Andricus fecundator* throughout development

In spring the bisexual generation form galls on the catkins (2-3mm). The autumn, unisexual generation form monolocular bud galls on *Q.robur* which resemble an enlarged bud (20-30mm). The larger bud gall was used for our investigation.

Few galls were available for this species, therefore the defined developmental stages are taken from only a limited number of samples. Fortunately each stage was obtained, although this was assessed by chamber and gall development, as larval dimensions could not be obtained. The stages are defined in the table below and schematic diagrams of the chamber are summarised in Figure 4.7.E. External and internal morphologies of the whole gall can be seen in chapter 5.

Table 4.6 The defined stages of development for *A.fecundator*

Stage of development	Chamber size	
	Length (mm)	Width (mm)
Stage 1	1.25 - 3.125	0.625 - 2.5
Stage 2	2.625 - 3.375	2.2 - 3.25
Stage 3	3.5 - 3.875	3.375 - 3.75
Stage 4	4 - 4.25	3.875 - 4.125

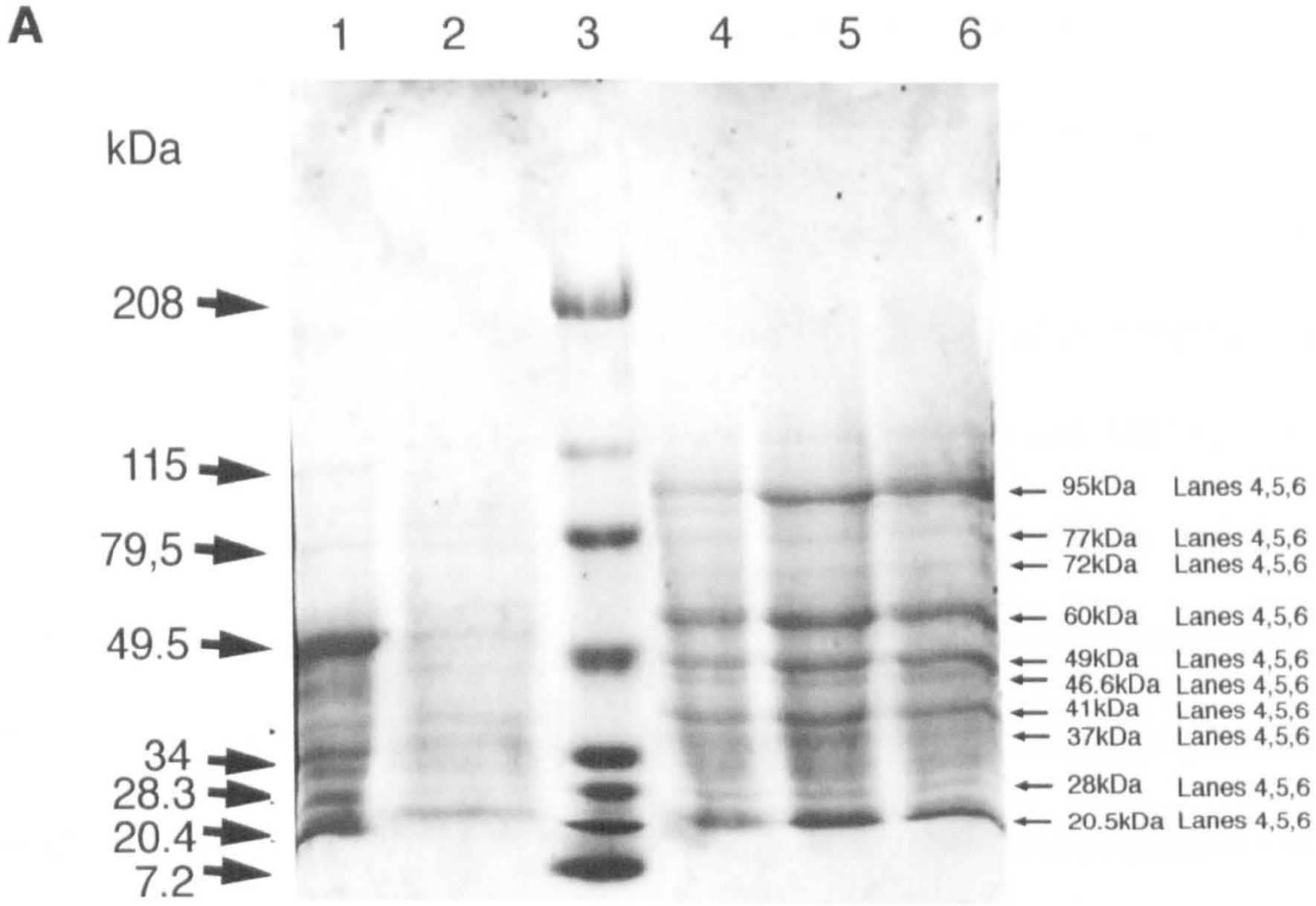
These protein signatures vary considerably from *A.quercuscalicis* as can be seen in Figure 4.12.A and Table 4.2, the signatures appear to vary little between stages. The most intense bands seen in all 3 stages are 95kDa, 60kDa, 49kDa and 41kDa. These are all expressed at equal levels throughout all stages. Additional bands which are less intense are 77kDa, 72kDa, 46.6kDa, 37kDa, 28kDa and 20.5kDa. These are all present in the 3 stages at equal intensities. The 77kDa and 20.5kDa bands are expressed in all the galls,

the 95kDa and 41kDa bands are also expressed in *C.quercusfolii* and *B.pallida*, the 46.6kDa band is also expressed in *A.quercuscalicis* and *N.quercusbaccarum* and the 37kDa band is shared with *C.quercusfolii*.

Figure 4.12.B shows the expression of the biotinylated protein in all 3 stages of development. All stages have equally intense signals, suggesting the biotinylated protein is expressed evenly throughout. In stage 3 only half the well was transferred onto the membrane, therefore, only half a band can be seen. Interestingly there is no 35kDa band detected by Coomassie staining, suggesting it is present in too low a concentration to be detected by Coomassie Brilliant Blue, but western analysis is more sensitive and can detect its presence. There is some non-specific binding in stage 2 where two faint bands at 41kDa and 55kDa can be seen. The molecular marker also shows non-specific binding, however, the acorn and the inner-gall samples clearly show specific signals at 35kDa.

4.4 Summary of protein expression in cynipid inner-gall tissue compared to acorn and leaf

Table 4.2 compares the protein signatures of the cynipid species tested here, acorn and oak leaf tissue. Within the gall signatures, only two of the visible bands are shared between all five species, at 77kDa and 20.5kDa, although several other bands are shared between two or three species. Interestingly, the 77kDa band present in all gall species is also present in the acorn but is not observed in the leaf signature. However, this does not indicate it is the



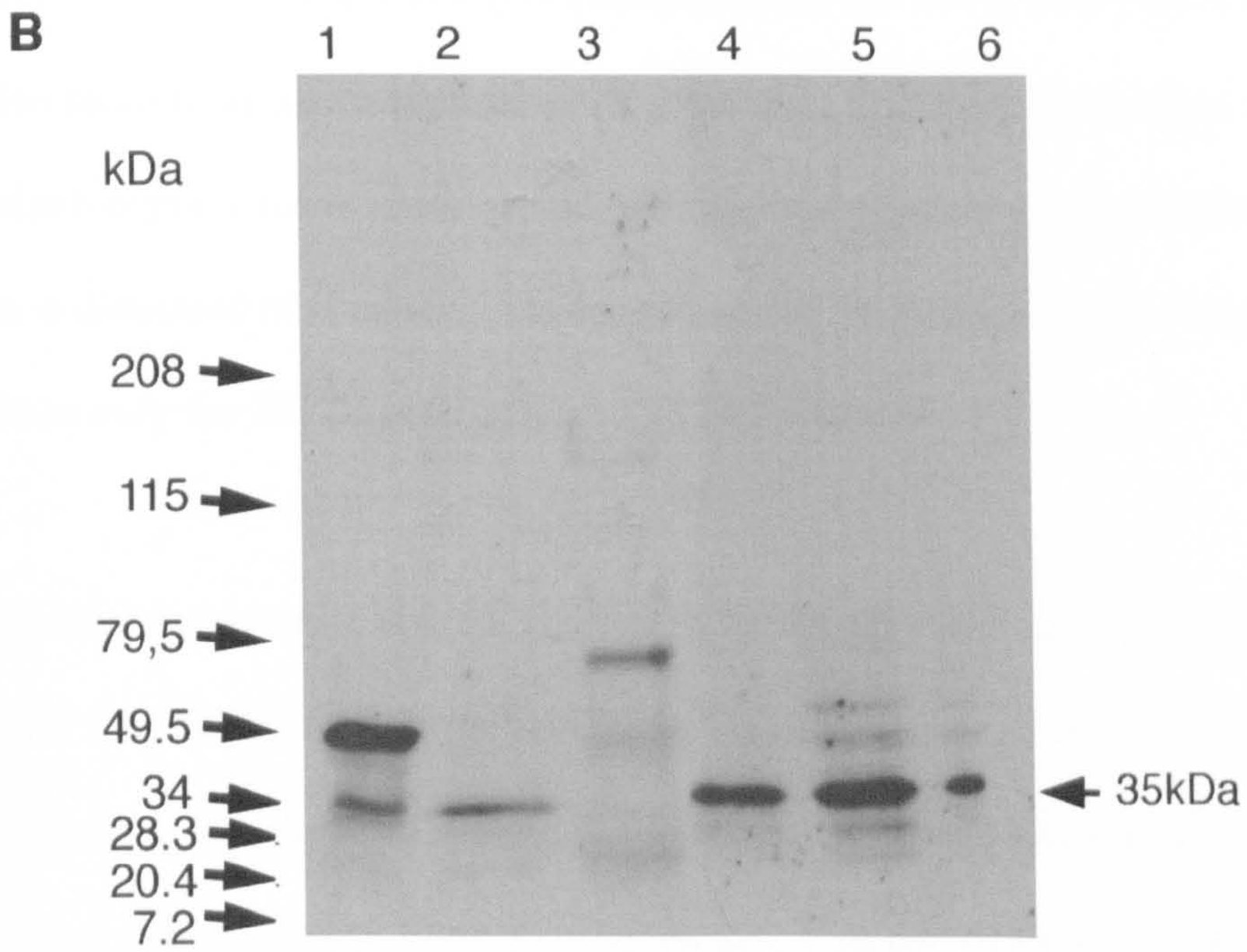


Figure 4.12 (A) SDS-PAGE gradient gel (6.5%-20%) and (B) western blot showing protein extracts from *A.fecundator* inner-gall tissue at 4 stages of development. In (B) the extracts were probed with streptavidin-horseradish peroxidase to analyse expression of biotinylated proteins. Lane1 oak leaf protein extract. Lane 2 acorn protein extract. Lane 3 contains the wide range molecular weight marker. Lane 4 20µg stage 1 *A.fecundator* inner-gall protein extract. Lane 5 20µg stage 2 *A.fecundator* inner-gall protein extract. Lane 6 20µg stage 3 *A.fecundator* inner-gall protein extract.

same protein in all these species and tissues, only the same molecular mass. Compared to *A.quercuscalicis* and *A.fecundator,* more proteins appear to be expressed in *B.pallida, N.quercusbaccarum* and *C.quercusfolii,* and some of these are common to all of the signatures. These three galls show a very similar pattern of development as is discussed in Chapter 5. Bands common to all three are 77kDa, 58kDa and 56kDa, of which 77kDa, 56kDa and 20.5kDa bands are also seen in the acorn signature. In addition to these, bands common to *B.pallida* and *C.quercusfolii* are 95kDa, 47.3kDa, 41kDa and 30kDa. The 41kDa band is also seen in the acorn extract. Bands common to *B.pallida* and *N.quercusbaccarum* are 38kDa and 35.4kDa, which are also present in the leaf signature. Only one band is common to *N.quercusbaccarum* and *C.quercusfolii* at 88kDa, which is also seen in the acorn signature. In contrast to this *A.quercuscalicis* and *A.fecundator* which express fewer proteins and, although they follow similar patterns of development, as is discussed in Chapter 5, the signatures of the two inner-gall tissue differ greatly and share only the 77kDa, 46.6kDa and 20.5kDa bands.

The acorn appears to share a number of bands with different gall species. The 88kDa band seen in the acorn protein signature is also expressed in *N.quercusbaccarum* and *C.quercusfolii*; the 77kDa band is common to all the species tested; the 56kDa band is shared with *B.pallida*, *N.quercusbaccarum*, *C.quercusfolii* and *A.quercuscalicis*; the 46.6kDa band is also expressed in *N.quercusbaccarum*, *A.quercuscalicis* and *A.fecundator*; the 41kDa band is shared with *B.pallida*, *C.quercusfolii* and *A.fecundator*; the 37kDa band is shared with *C.quercusfolii*, and *A.fecundator*; the 35.4kDa is shared with *B.pallida* and *N.quercusbaccarum*; the 27.3kDa band is shared with *C.quercusfolii*; and the 24.35kDa band is shared with *B.pallida*.

Although these protein signatures only represent the segregation of total protein content according to molecular mass, the several bands shared between the gall tissue and acorn is interesting as gall formation has been speculated to use seed developmental pathways during development (Schönrogge *et al.*, 2000). Whether the protein signature is an indication of the signals being used to control gall formation remains to be determined, but the identification of these proteins, in particular the ones common to a number of cynipid species and acorn, will extend our understanding of the proteomics of the gall. The confirmation of similar protein expression in gall tissue and acorn protein extracts, will take us one step closer to determining whether gall formation does involve seed developmental pathways. The formation of a seed-like structure by cynipids would ensure nutrients for the developing larva and would mean the insect can hijack existing developmental pathways of the host, to use for its own advantage. If this is the pathway used by cynipids, the elucidation of this reprogramming, to induce seed development in novel positions on the host, would provide opportunity to extend our understanding of seed development signalling pathways, in addition to gall development.

Sequence analysis of the shared protein, including the biotinylated protein, will demonstrate the extent of similar protein expression between gall tissue and seeds, and also confirm the identity of BCCP. I have shown here a 35kDa protein common to seeds and gall tissue, which is differentially expressed in several gall species throughout development. From the identification of additional proteins, the signals secreted by the larvae to induce the expression of the selective proteins and therefore maintain the development of gall formation will hopefully be elucidated.

5: Cell organisation throughout gall development

5. 1 Cell biology of galls

Cytological differences between inner-gall and non-gall tissues such as increased cytoplasm, fragmented vacuole, high lipid content, enlarged nucleus and nucleolus were discussed in chapter 1. These cytological changes occur in response to the signalling molecule from the larva and the cell organisation is dramatically altered from normal plant development. The significant changes demonstrate the extent of regulatory control the larva has over cell proliferation, organisation and differentiation of specific cell types. The whole process is tightly regulated and by elucidating these signalling pathways our understanding of both plant development and gall formation will be greatly enhanced.

An additional cytological variation of particular interest is the increase in nuclear size and alteration in chromosome structure, with some inner-gall cells becoming polytene. Polytene chromosomes result from multiple rounds of endoreduplication, DNA replication without nuclear division, to form polyploid cells which then become polytene when the sister chromatids are organised alongside each other and held together at the centromere or along the chromosome, appearing as a cable-like structure (Nagl, 1981). This has been noted in a number of gall species and the copy number is believed to decrease away from the larva (Hesse, 1968). This observed pattern of genome copy number suggests that the larva is either in direct control of the cell cycle or an indirect affect of the signal is multiplication of the genome. Either way, the signal is significantly redirecting the normal cell cycle to adapt to the larval needs. The

alteration is a fascinating characteristic of gall formation and, in an attempt to understand further the control the larva has over the host and develop a possible marker in the bioassay, I decided to investigate the changes in cell organisation and chromosome structure in several species of gall at different stages of development.

5.1.1 The plant cell cycle

The occurrence of polytene chromosomes is seen elsewhere in plants, and is a specific characteristic of particular cell types. A summary of the plant cell cycle in relation to polytene cells, will now be discussed to introduce my research into the cell organisation and cytological characteristics of gall tissue, using tissue sections, protoplast preparations and *in-situ* hybridisation.

Cell division in plants occurs in three main areas: the shoot apical meristem, root apical meristem and the vascular cylinder (Jacobs, 1992). These undergo localised cell divisions providing new cells for radial expansion, shoot and root elongation, and cells for new organs such as leaf and flower development. The identity of a plant cell depends on position and the neighbouring cell type (Loidl and Loidl, 1996). Extensive cell cycle research has been carried out mainly on yeast and animal cells; however, over the last decade the control over the cell cycle in higher plants has become of great interest to plant biologists. The regulatory controls taking the cell through this cycle are conserved throughout plants, animals and yeast and involve many complex and tightly regulated interactions. I will summarise briefly the relevant control mechanisms used by plants to achieve a successful cell cycle and the consequences when the regulation is disrupted, especially with respect to endoreduplication and polytenisation.

The cycle involves four stages: G1 (gap 1), S phase (DNA synthesis), G2 (gap2) and M (mitosis). Temporal association of cyclin-dependent protein kinases (Cdks) and the regulatory proteins, cyclins, ensure passage through the checkpoints of each phase at the correct time. This is achieved by an increase in the concentration of appropriate cyclin proteins, and ubiquitin-mediated proteolysis of the complex after entry into the new phase to allow passage into the next. The passage from G1 into S phase is the start of the cycle and decides if the cell will continue dividing or enter G0 and differentiate. The initiation of S phase in humans requires $p34^{cdk2}$ complexed with A and E cyclins (Krude *et al.*, 1997). S phase kinases are thought to phosphorylate replication proteins which activate DNA replication (Grafi, 1998). The cyclins A and E increase in concentration and activate the protein kinases which then phosphorylate appropriate substrates to initiate the start of S phase (Grafi, 1998). It has been demonstrated in maize that S phase related kinases are required for entry into S phase (Grafi and Larkins, 1995). Once the cell has reached the end of S phase, then the transition to G2 is thought to be controlled by a cdk2-cyclin A complex (Grafi, 1998). In G2 the cell either arrests or continues into mitosis. Interestingly, a decrease in expression of cyclin A is coupled with the onset of endoreduplication in *Drosophila*, which can also be inhibited if cyclin A is continually present (Weiss *et al.*, 1998). The attachment of ubiquitin to cyclins and the subsequent destruction ensures the cell can move through to the following phase. If the ubiquitin-mediated proteolysis of the cyclin is prevented, as demonstrated in yeast by (Singer *et al.*, 1996), then the cell undergoes incomplete replication of the DNA and the level of DNA increases.

The G2-M checkpoint controls whether the cell undergoes division and is important for understanding endoreduplication. Mitosis is controlled by maturation promoting factor (MPF), which is a complex of $p34^{cdk2}$ and cyclin B. Levels of cyclin B increase at the end of G2 and bind to the protein kinase, which then phosphorylates unknown substrates and induces mitosis. The high concentration of the $p34^{cdc2}$ cyclin B complex also inhibits DNA synthesis, ensuring the correct temporal order of the phases (Hayles, 1994).

In the case of endoreduplication, the regular cell cycle is disrupted to enable the cell to increase the amount of DNA. This can be achieved by multiple initiations during the same S phase, multiple rounds of S phase or many rounds of S phase and G2 without mitosis (Grafi, 1998). Examples of endoreduplication can be seen in mammals, plants and insects and occurs mainly in cells which are under high metabolic activity (Grafi and Larkins, 1995). The induction of endoreduplication in maize endosperm has been shown to be a result of an inhibition of MPF and induction of S phase kinases (Grafi and Larkins, 1995). A number of reagents are known to inhibit mitosis and cause alterations to the surface of cells, however, the exact signal inducing endoreduplication in maize endosperm and other endoreduplicated cells remains unknown (Grafi and Larkins, 1995).

Polyploid and polytene cells have been noted in cynipid galls and many other types of galls formed by bacteria (Therman, 1995; Kodama, 1974; Butcher *et al.*, 1975), fungi (Guseinov and Vanyshin, 1975; Callow, 1975), gall mites (Hesse, 1968, 1971, 1972; Westphal, 1974), gall midges (Garrigues, 1966; Hesse, 1968, 1973) and Homoptera

(Anders, 1955, 1960). A study carried out by Hesse (1968) using DNA-cytophotometric measurements, revealed that most cynipid galls tested had endopolyploid or polytene nuclei, the greatest being *in Andricus marginalis* which had 1024n nuclei in the nutritive cells (Hesse, 1968). It is believed that the increase in genome copy number ensures that the cell is able to cope with the increased metabolic activity. In plants, polytene chromosomes are normally seen in cells such as (i) the antipodal cells, a group of three cells derived from the mother cell at the basal end of the plant embryo sac, (ii) the nutritive endosperm surrounding the embryo, (iii) the suspensor cells, arising from the basal cells of the proembryo and providing nutrients to the embryo and (iv) anther tapetal cells lining the cavity of the anther and providing nutrients to maturing pollen grains. Note that inner-gall cells also provide nutrients for the developing larva. The development and function of these cells have been studied in all the different tissues, although the trigger to induce polytenisation remains unknown. As with the inner-gall cells, the demand on these polytene cells is high, and one mechanism used to cope with demand is to increase the amount of DNA enabling a high level of transcription. Unlike *Drosophila* salivary gland polytene chromosomes, the plant polytene chromosomes are not paired and they do not show the puffing and banding pattern seen in *Drosophila*. The plant polytene chromosomes, including those in galls, are present in diploid number and can form several conformations. This include the looping out of some of the chromatids, the formation of a bow-tie shape as all the chromatids fan out from the centromere or a more compact structure when all of the chromatids lie together (Nagl, 1981).

5.1.2 Interruption of the cell cycle by nematodes

Other biotic-plant interactions, such as the nematode-plant interaction, demonstrate the induction of cytological changes, including cell cycle modifications and enlarged multinucleate feeding cells (Engler *et al.*, 1999). The root knot nematode and the cyst nematode induce giant cells and syncytia respectively, as elaborate feeding cells in the nematode-plant interaction. The formation of the enlarged feeding cells could be compared to that seen in cynipids as the inner-gall cells are essentially feeding cells induced by the larva to enabling the production of sufficient nutrients. Both nematode and gall feeding cells are greatly enlarged with an increase in DNA content, cytoplasmically dense, fragmented vacuoles and high metabolic activity to carry out the nutritive role in the parasite-plant interaction. As with cynipids, if the nematode is removed or killed, the giant cells stop developing, demonstrating here too a constant stimulus from the nematode is required. Once the nematode feeding cells have reached a certain DNA content, they stop increasing the amount of DNA, indicating that they have amplified sufficiently to reach demand (Engler *et al.*, 1999). In cynipid nutritive tissue, the cells close to the larva have the highest amplification, and moving distal from the larva this decreases. The cells furthest from the larva will be under less demand and do not supply the larva directly with nutrient, explaining their lower DNA level. The nematode-host interaction may help understand how the cynipid larva is disrupting the normal host cell cycle. Although the nematode feeding cells are multinucleate and do not show polytene chromosomes, multiple rounds of endoreduplication do occur within each of the nuclei. This system has been well studied, and the differential gene expression of the feeding cells is in the process of

being analysed (Bird *et al.*, 1996; Koltai and Bird, 2000; McCarter *et al.*, 2000). These feeding cells are formed from cells which would normally become xylem cells and characteristics similar to xylem cells can be noted in the feeding cells. It has been suggested, therefore, that the feeding cell induction, although still poorly understood, may use signalling pathways similar to xylem formation (Bird, 1996). The mature feeding cells do not form mature xylem so the complete signalling pathway is clearly not used (Bird, 1996). Both feeding cells are multinucleate; however, the process by which they each achieve this differs. To make the feeding cells, the root knot nematode interrupts the normal cell cycle by inducing consecutive mitoses without cell division and ploidy levels also increase by rounds of endoreduplication. Syncytia induction by the cyst nematode also involves some cycles of endoreduplication but the multinucleate state arises mainly by cell wall dissolution from neighbouring cells. The cell cycle patterns of both these specialised feeding cells have been analysed using cell cycle inhibitors, molecular markers and DNA synthesis (Engler *et al.*, 1999). These showed that DNA synthesis and mitosis is essential in the development of the giant cells and syncitia. If either of these are blocked, then they do not fully develop. Demonstrating, therefore, in the case of the giant cell development that cycles of endoreduplication are not sufficient and mitosis is required (Engler *et al.*, 1999). In developed giant cells, very little mitosis or endoreduplication is seen which suggests that the DNA amount is increased until the cell contains sufficient to maintain high metabolic activity. The syncitia are less affected when mitosis is blocked as the multinucleate state is mainly achieved by cell wall dissolution, however, it prevents the feeding cell from developing fully as the radial expansion is prevented by the block on division of neighbouring cells (Engler *et al.*, 1999). Analysis of the host gene

expression within the giant cells has been carried out (Bird, 1996; Koltai and Bird, 2000; McCarter *et al.*, 2000) to analyse the genes induced or up regulated by the nematode. Further identification of gene expression will aid the elucidation of the mechanism used by the nematode to alter the host development. To date a number of genes have been identified within the feeding cells, one being a Tkn2 KNOX gene which is a transcriptional regulator necessary for the normal development of the meristem (Bird and Koltai, 2000). When the KNOX gene is overexpressed then the same phenotype occurs as a disruption of polar auxin transport (Tsiantis *et al.*, 1999). During giant cell formation there is an increase in auxin, which could be caused by the disruption of auxin transport as a result of KNOX expression (Tsiantis *et al.*, 1999). Alternatively, it could be caused by flavonoids as discussed in Chapter 1.

5.1.3 Arabinogalactan proteins as cell specific markers

As introduced in chapter 1, AGPs are a group of proteoglycans which are believed to have roles in cell-cell signalling, cell proliferation and development. The signalling properties and the differential expression within specific cell types at particular stages of development mean AGPs are essential for the elucidation of cell-cell signalling and development. In galls, cell-cell signalling and cell specific markers would help our understanding of the process and enable us to follow the cytological changes during gall initiation and development. Three antibodies against AGPs were obtained to investigate AGPs in gall formation. JIM13, JIM4 and MAC207 were provided by Dr Keith Roberts. Although little is known about the carbohydrate epitopes, JIM4 binds to less common AGPs whereas JIM13 and MAC207 bind to common AGPs (Nothnagel, 1997). As discussed in chapter 1, JIM13 is expressed in cells which become xylem

cells. MAC207 epitope is present in a number of AGPs, although the expression of these has been shown to disappear in tissues involved in sexual reproduction and can not be detected in early zygotic embryo development. The expression does occur later in embryo development, after the heart stage (Pennell *et al.*, 1989). JIM4 has been shown to bind to AGPs secreted in exudates gums (Yates *et al*, 1996). The use of these on gall tissue will be discussed in section 5.6.

5. 2 How do larvae control plant development?

Further understanding of the control cynipid larvae have over the host plant cells, and the signals used to alter the development, is important for the elucidation of gall formation and plant signalling. To achieve this a detailed study of cytological changes within the host, in response to the larva, was carried out, to try and determine how the larva is controlling the host's development to achieve gall formation. To investigate cytological changes, cell organisation, cellular activity and cell specific markers were analysed throughout gall formation using tissue sections from several species of developing galls. Immunohistochemistry was used on the tissue sections to investigate the spatial distribution of putative BCCP throughout gall tissue. Chromosome alterations were investigated by fluorescent *in-situ* hybridisation (FISH) and protoplast analysis at different stages of development. Cell specific markers were analysed using AGP antibodies JIM4, JIM13 and MAC207 on tissue prints of the galls in an attempt to detect cell surface markers, possibly indicative of cell type. The majority of this work was presented at "*The Plant-Biotic interactions Keystone Symposium 2000*", and published as an abstract in the proceedings (see appendix).

5.3 Cell organisation throughout gall development

Oviposition by the adult female is soon followed by many host cytological changes, as discussed in Chapter 1. To investigate these changes in a variety of cynipid galls, collections were taken at weekly intervals from the first appearance of the gall. The outer-gall was removed and the remaining chamber fixed in formaldehyde, washed in PBS and embedded in Tissue-tek (Agar Scientific), before taking cryosections of the gall chamber (see section 2.4.1.3). The inner-gall was used for sectioning as the outer-gall consists mainly of parenchyma and once formed little change occurs, except for cell expansion during the growth phase of development (see Chapter 1). The nutritive inner-gall tissue is common to all cynipid galls and is an essential tissue for larval survival, therefore, comparison of several cynipid galls in the production of the same tissue may indicate if similar initial signals are being used, and at what stage these may differ. To analyse the inner-gall organisation, tissue sections of 15-20µm taken throughout the defined stages of development were stained with 10% DAPI to stain the DNA, and analysed using a Leitz Aristoplan fluorescent microscope.

5.3.1 Biorhiza pallida development

B.pallida has two generations a year forming on *Q.robur*, as discussed in chapter 4. I chose the bisexual generation multiocular bud gall for this investigation due to its abundance and accessibility. The development of *B.pallida* has been well studied as discussed in chapter 1, therefore, I decided to use this and less studied galls to compare developmental patterns. *B.pallida* will be discussed first.

Stage 1

The eggs are oviposited between the bud scales of *Q.robur* and placed on the apical meristem where development begins with bud burst. The cells below the egg lyse and the cells around it begin to divide and from the larval chamber. Soon after gall formation begins, the gall is visible as a red ball pushing through the bud scales. It is at this point first collections were made. Figure 5.1 shows the external and the internal morphologies of the gall in (A) and (B), and tissue sections of the gall at stage 1 can be seen in (C) and (D). This stage represents the end of the initiation phase where the chambers have formed, remain extremely small and very little inner-gall tissue surrounds the larva. The spongy outer-gall has formed but, again, remains small. The chambers make a fan shape fanning out from the bottom of the gall with little space between the 30-40 individual chambers. The tissue sections (20μm) of the chamber in (C) and (D) show that the inner-gall cells remain small, they are tightly packed and appear yellow in colour, suggesting the lipid content of the cells is high.

Stage 2

During the growth phase, cell expansion occurs and the outer cortex and the inner chambers increase significantly in size. Figure 5.1 shows the external (E) and internal (F) morphologies of the gall at this stage. A tough layer of sclerenchyma forms around the chamber thought to provide support and protection to the larva. Tissue sections (15μm) in (G) and (H) of Figure 5.1 show the growth of the chamber. The cells are less compact and the cells immediately around the larva are larger than those in the outer part of the chamber. The cells immediately surrounding the larva, shown in (H), have increased in size and the nucleus and nucleolus have enlarged compared to the previous stage (D).

Stage 3

Figure 5.2 shows the external (A) and internal (B) morphologies at this stage. Externally the gall remains similar size to the previous stage but the pink colouring turns brown in colour. Internally the chambers increase slightly in size and the larvae continue to develop and graze on the thick layer of inner-nutritive cells. The tissue sections (20μm) in (C) and (D) show the inner-nutritive cells are clearly visible. Immediately surrounding the larva are 3 layers of the enlarged lipid filled cells with an enlarged nucleus and nucleolus, and the smaller nutritive parenchyma cell surround these in the outer region of the gall. The enlarged cells appear to have polytene nuceli, which will be analysed and discussed in section 5.5.1. The parenchyma is transformed into the enlarged, lipid filled inner nutritive cells as the larva grazes, providing nutrients to each larva.

Stage 4

The external (E) and internal (F) morphologies in Figure 5.2 show the mature gall where all the inner-gall tissue has been grazed and only the sclerenchyma capsule remains and the larva is ready to pupate. Tissue sections in Figure 5.2 (G) and (H) show the thin layer of inner-gall cells and the sclerenchyma capsule. There is little parenchyma between the chambers and the sclerenchyma layers back onto each other as can be seen in (H).

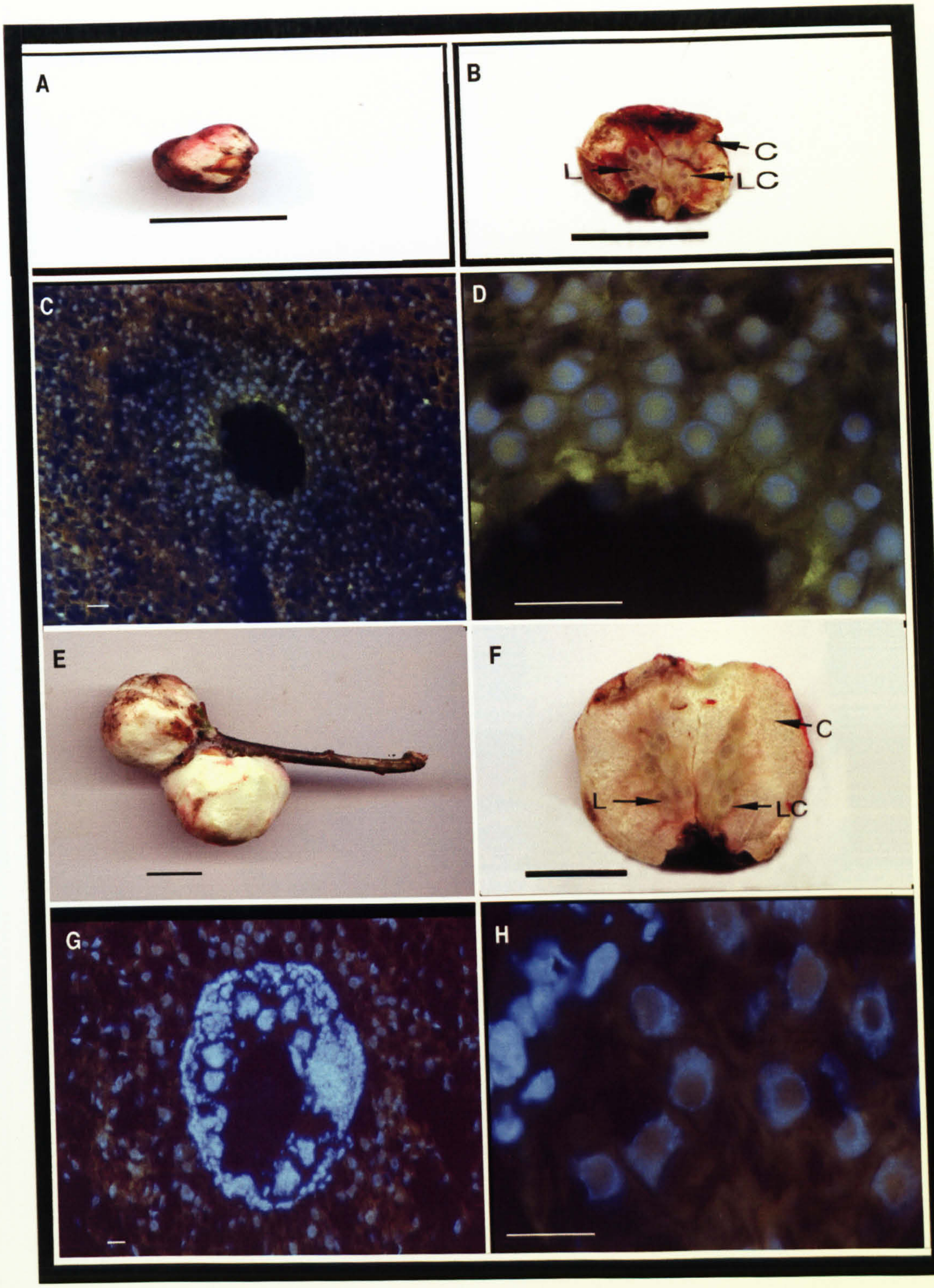


Figure 5.1 *B.pallida* at stage 1 and stage 2 of development. (A) External gall morphology at stage 1. (B) Internal gall morphology at stage 1. (C) Tissue section of the larval chamber at stage 1 stained with DAPI. (D) Tissue section of inner-gall cells at stage 1 stained with DAPI. (E) External gall morphology at stage 2. (F) Internal gall morphology at stage 2. (G) Tissue section of the larval chamber at stage 2 stained with DAPI. (H) Tissue section of inner-gall cells at stage 2 stained with DAPI. LC = Larval chamber, C = Cortex
A, B, E, F, scale bar = 1cm. C,D,G,H scale bar = 50μm

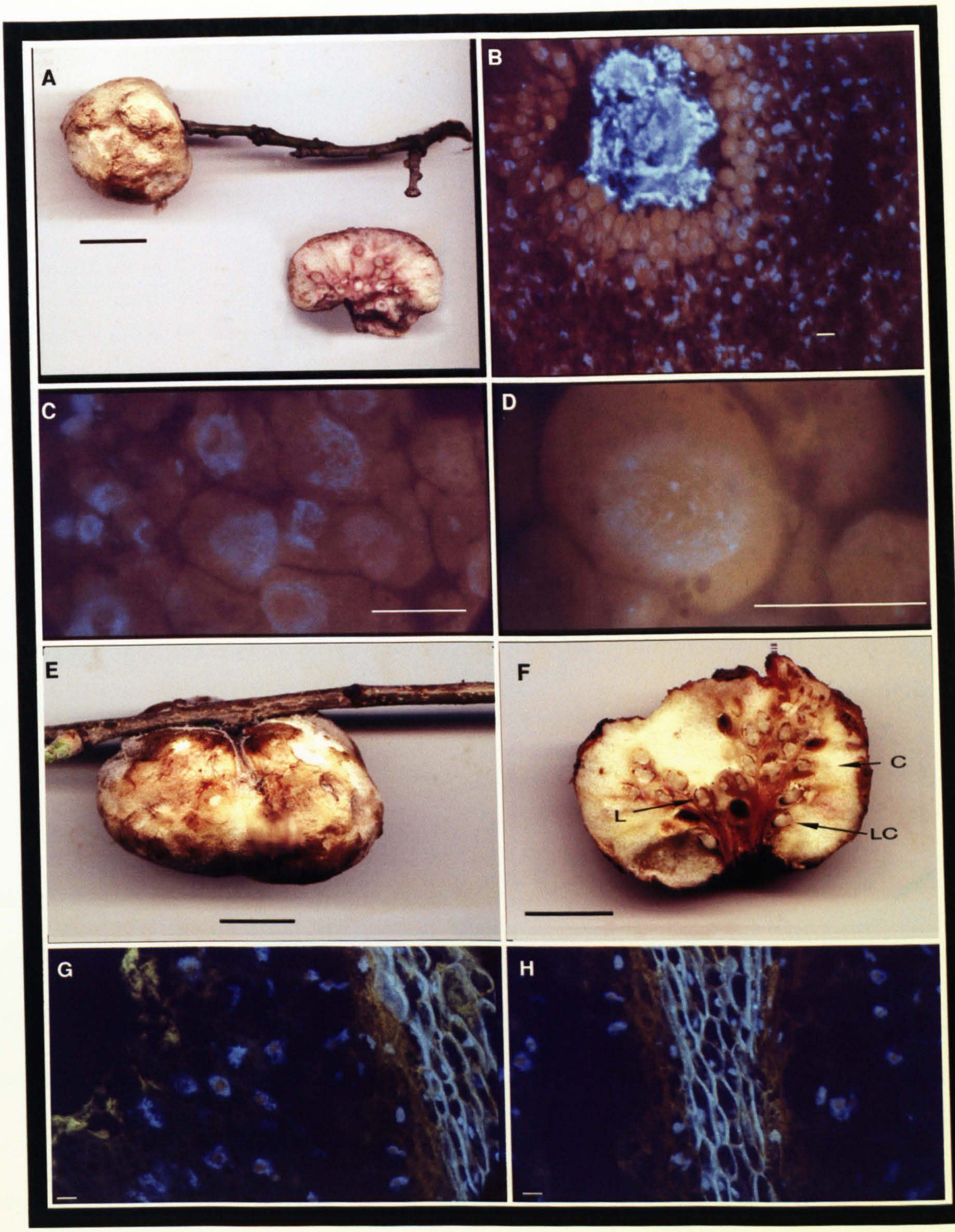


Figure 5.2 *B.pallida* at stage 3 and stage 4 of development. (A) External gall morphology at stage 3. (B) Internal gall morphology at stage 3. (C) Tissue section of larval chamber at stage 3 stained with DAPI. (D) Tissue section of inner-gall cells at stage 3 stained with DAPI. (E) External gall morphology at stage 4. (F) Internal gall morphology at stage 4. (G) Tissue section of the larval chamber at stage 4 stained with DAPI. (H) Tissue section of 2 chambers at stage 4 stained with DAPI.
A, B, E, F scale bar = 1cm. C, D, G, H = scale bar = 50μm

5.3.2 Neuroterus quercusbaccarum development

Like *B.pallida, N.quercusbaccarum* has two generations a year, as discussed in chapter 4. The spring leaf galls were available from our chosen field sites and were chosen for this investigation.

Stage 1

Early in leaf expansion *N.quercusbaccarum* begin to form as a small, glassy, spherical gall, containing a single larva in a spherical chamber. Figure 5.3 shows external (A) and internal (B) morphologies and tissue sections (20μm) in (D) and (E) at stage 1. This represents the end of the initiation phase and the cortex has formed. In the early stages there is little inner-gall tissue and spongy parenchyma making up the outer gall. The tissue sections show that there are 3-4 layers of nutritive tissue present around the larva, which is more advanced than seen in *B.pallida*. The cells appear enlarged with an endoreduplicated and possibly polytene nucleus and nucleolus. This is a monolocular gall and the larvae and chamber sizes are larger than *B.pallida.* The larger larva may explain the thicker layer of nutritive cells at an earlier stage, or this gall could be slightly more advanced in development than that observed in *B.pallida*. As in *B.pallida* there is no real distinction between the chamber and the cortex, only the different cell types.

Stage 2

Figure 5.3 shows external (E) and internal (F) morphologies at this stage. The size of the outer-gall and the inner-chamber both increase in size. More inner-gall tissue forms around the larva forming a more distinct chamber. No sclerenchyma forms between the inner tissue and the outer tissue. Tissue sections (15μm) in (G) and (H) of Figure 5.3 show

3-4 layers of the enlarged inner-nutritive cells around the larva, which appear lipid–filled and have enlarged nuclei and nucleoli, clearly seen in (H). Compared to *B.pallida* at this stage the total quantity of inner-gall tissue within the chamber appears much more and the cells are more compact.

Stage 3

Externally the gall remains a similar size to the previous stage, as shown in Figure 5.4 (A). Internally the chamber increases in size and the amount of nutritive tissue lining the chamber increases significantly (Figure 5.4 (B)). As in *B.pallida,* the larva grazes on the inner-nutritive cells and continues to grow and develop. The tissue sections in Figure 5.4 (C) and (D) show the enlarged inner-gall cells. The number of layers (5-6) of inner-nutritive cells is greater than *B.pallida,* probably because the single chamber of *N.quercusbaccarum* is larger than the chambers in *B pallida* and the *N.quercuscalicis* larvae are larger. The first layer of cells show anticlinal division and the layers behind this periclinal division. In the outer parts of the chamber, the cell size and nuclei do decrease in size, as in *B.pallida.* In *B.pallida* the larva increase in size to fill the available space and eventually fill the whole chamber when grazing is complete, however, *N.quercusbaccarum* do not fill the chamber and appear to move around the chamber more frequently. When grazing is complete, they are ready to pupate.

Stage 4

No sections are available for this stage. As in *B.pallida*, the inner-gall tissue is grazed and the larva reaches maturity and pupates. No sclerenchyma layer is formed in *N.quercusbaccarum*.

5.3.3 Cynips quercusfolii development

C.quercusfolii has two generations a year, as discussed in chapter 4, and the autumn gall was used for this investigation due to availability.

Stage 1

The spherical gall begins to form on the underside of leaves in July. Figure 5.5 shows the external and internal morphologies (A) and (B) late in stage 1. Internally the larval chamber is small with a thin layer of inner-gall tissue. The spongy outer cortex has formed but still remains small. In comparison to *N.quercusbaccarum* the cortex is much thicker and the chamber size larger. Figure 5.5 (C) shows a tissue section (20μm) of the larval chamber. Only a fraction of the larval chamber can be seen due to the large chamber size. There is only a layer of enlarged cells lining the chamber, which appear yellow and are probably high in lipids.

Stage 2

The gall cortex and larval chamber both expand and more layers of inner-gall cells line the chamber, shown in Figure 5.5 (E) and (F). Tissue sections (15μm) of the larval chamber, shown in (G) and (H), show the cells are less compact and have increased in size. The layers of nutritive inner-gall cells are fewer and less compact than seen in *N.quercusbaccarum*, which is surprising as *C.quercusfolii* is larger than *N.quercusbaccarum*. The cells do appear enlarged with enlarged nuclei.

Stage 3

The gall does not increase in size but enters the maturation phase, the chamber increases and the inner-gall tissue is grazed by the larva. No sclerenchyma layer forms

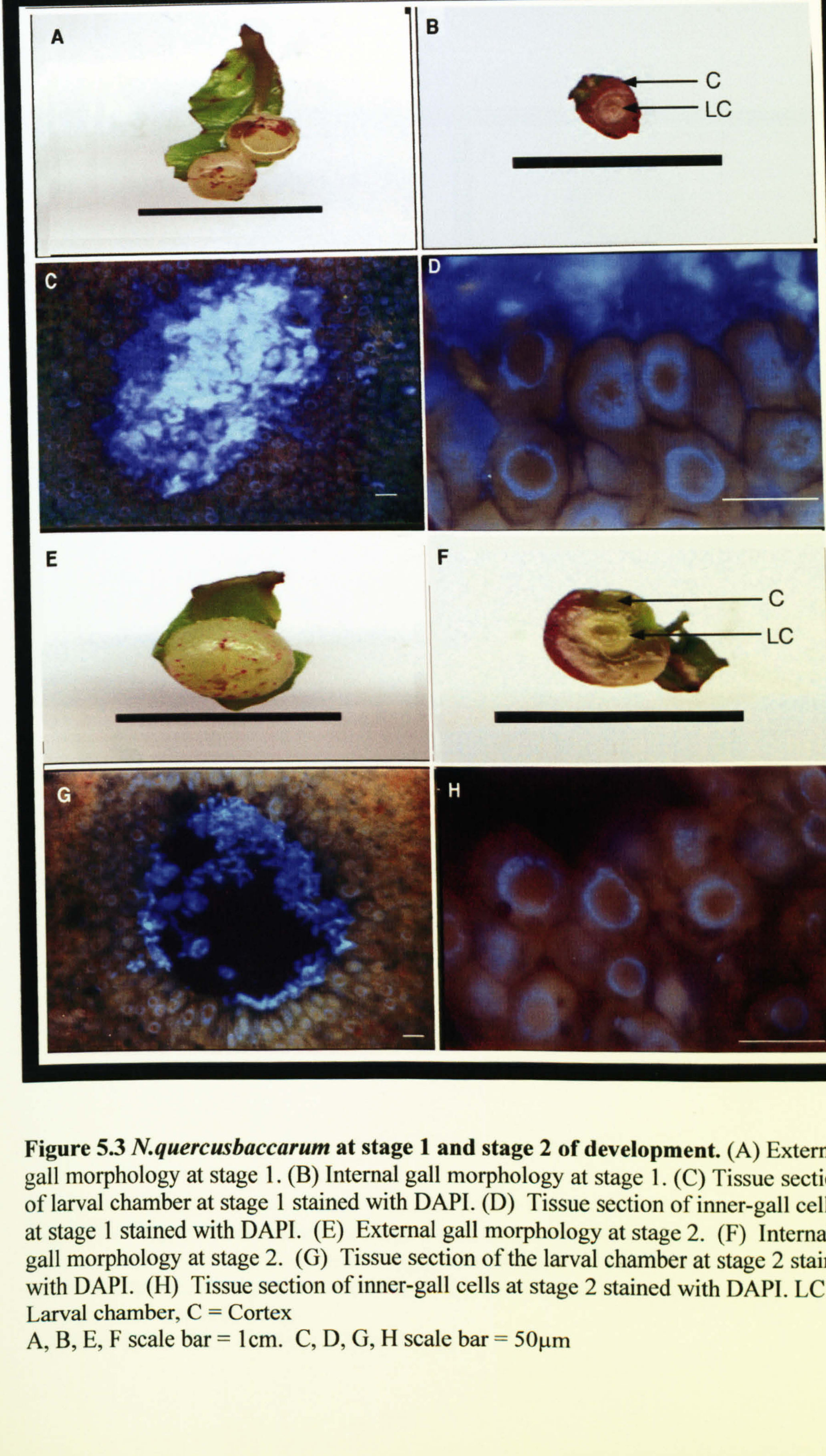


Figure 5.3 *N.quercusbaccarum* at stage 1 and stage 2 of development. (A) External gall morphology at stage 1. (B) Internal gall morphology at stage 1. (C) Tissue section of larval chamber at stage 1 stained with DAPI. (D) Tissue section of inner-gall cells at stage 1 stained with DAPI. (E) External gall morphology at stage 2. (F) Internal gall morphology at stage 2. (G) Tissue section of the larval chamber at stage 2 stained with DAPI. (H) Tissue section of inner-gall cells at stage 2 stained with DAPI. LC = Larval chamber, C = Cortex
A, B, E, F scale bar = 1cm. C, D, G, H scale bar = 50μm

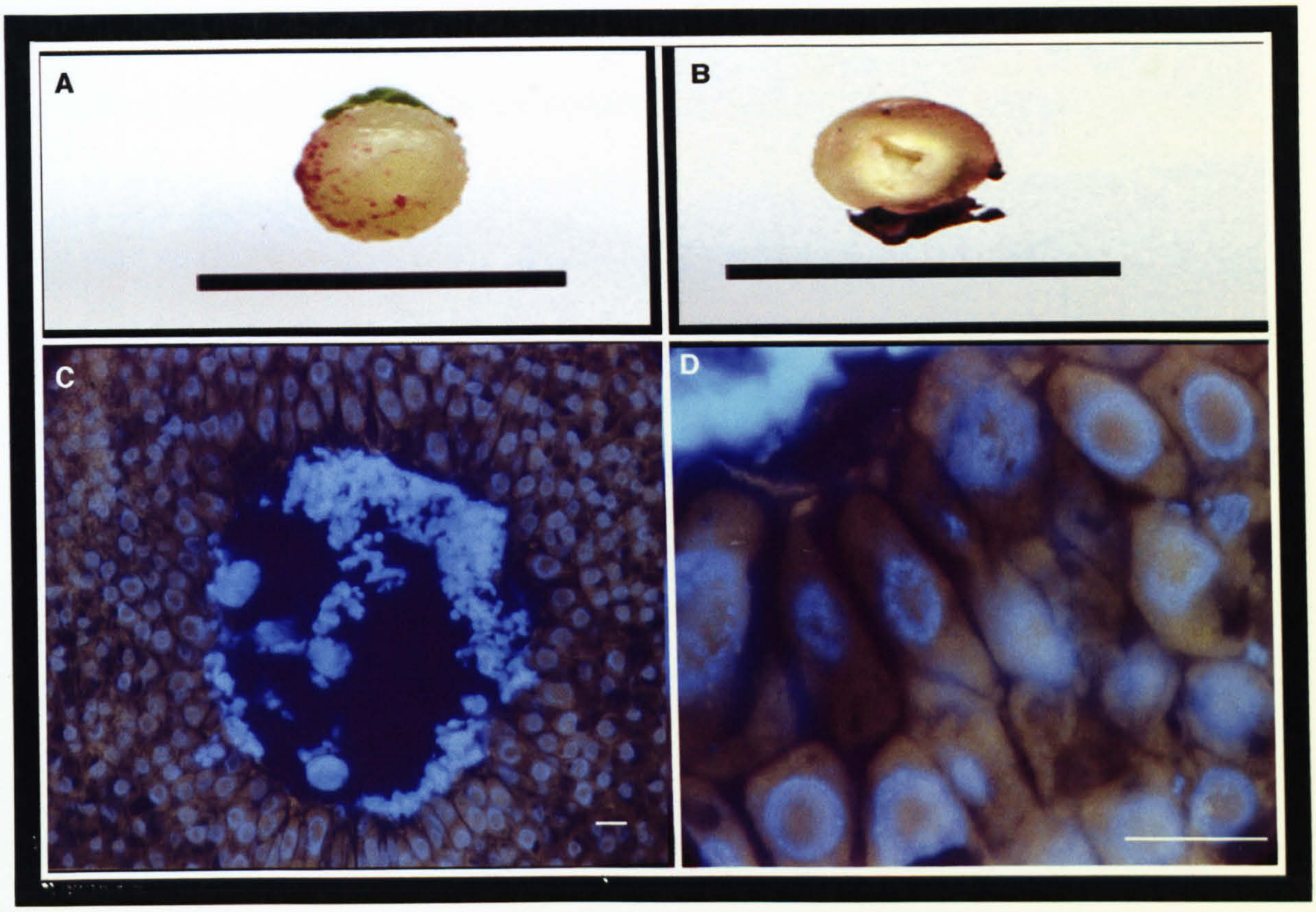


Figure 5.4 *N.quercusbaccarum* at stage 3 and stage 4 of development. (A) External gall morphology at stage 3. (B) Internal gall morphology at stage 3. (C) Tissue section of larval chamber at stage 3 stained with DAPI. (D) Tissue section of inner-gall cells at stage 3 stained with DAPI.
A,B scale bar = 1cm. C, D scale bar = 50μm

C.quercusfolii has two generations a year, as discussed in chapter 4, and the autumn gall was used for this investigation due to availability.

Stage 1

The spherical gall begins to form on the underside of leaves in July. Figure 5.5 shows the external and internal morphologies (A) and (B) late in stage 1. Internally the larval chamber is small with a thin layer of inner-gall tissue. The spongy outer cortex has formed but still remains small. In comparison to *N.quercusbaccarum* the cortex is much thicker and the chamber size larger. Figure 5.5 (C) shows a tissue section (20μm) of the larval chamber. Only a fraction of the larval chamber can be seen due to the large chamber size. There is only a layer of enlarged cells lining the chamber, which appear yellow and are probably high in lipids.

Stage 2

The gall cortex and larval chamber both expand and more layers of inner-gall cells line the chamber, shown in Figure 5.5 (E) and (F). Tissue sections (15μm) of the larval chamber, shown in (G) and (H), show the cells are less compact and have increased in size. The layers of nutritive inner-gall cells are fewer and less compact than seen in *N.quercusbaccarum*, which is surprising as *C.quercusfolii* is larger than *N.quercusbaccarum*. The cells do appear enlarged with enlarged nuclei.

Stage 3

The gall does not increase in size but enters the maturation phase, the chamber increases and the inner-gall tissue is grazed by the larva. No sclerenchyma layer forms

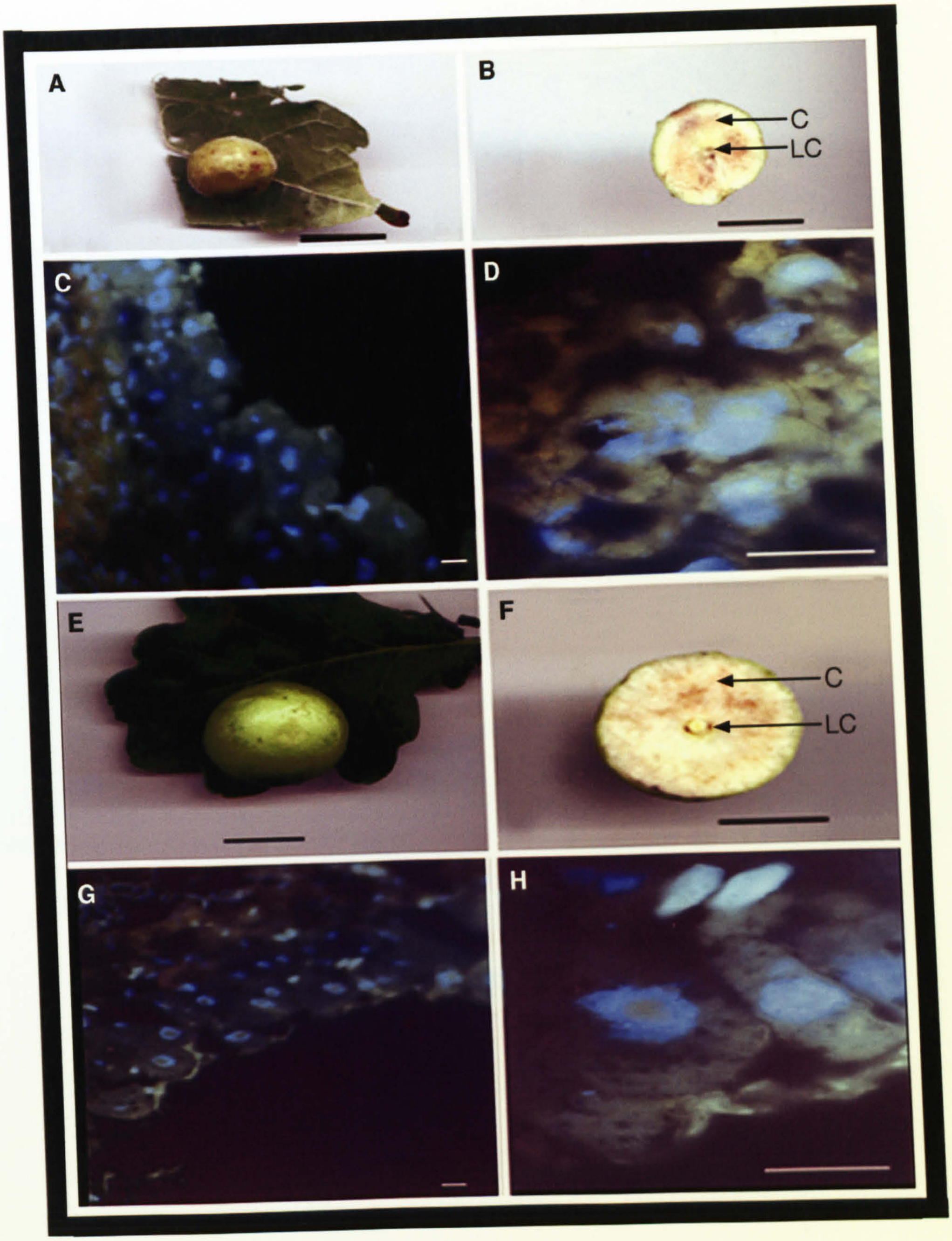


Figure 5.5 *C.quercusfolii* at stage 1 and stage 2 of development. (A) External gall morphology at stage 1. (B) Internal gall morphology at stage 1. (C) Tissue section of larval chamber at stage 1 stained with DAPI. (D) Tissue section of inner-gall cells at stage 1 stained with DAPI. (E) External gall morphology at stage 2. (F) Internal gall morphology at stage 2. (G) Tissue section of larval chamber at stage 2 stained with DAPI. (H) Tissue section of inner-gall cells at stage 2 stained with DAPI. LC = Larval chamber, C = Cortex

A, B, E, F scale bar = 1cm. C, D, G, H scale bar = 50μm

Figure 5.6 *C.quercusfolii* at stage 3 of development. (A) External gall morphology at stage 3. (B) Internal gall morphology stage 3.
A, B scale bar = 1cm.

around the larval chamber. Figure 5.6 shows the external (A) and internal (B) morphology of the gall at this stage. Tissue sections of this stage are not available. A large chamber remains, encapsulating the larva, which does not fill the whole of the larval chamber, unlike the galls discussed here. The larva pupates and over winters in the gall, emerging in spring to lay eggs for the spring generation bud gall.

5.3.4 Diplolepis rosae development

This gall has only one generation each year and forms hairy, multilocular galls on the buds, leaves or stem of *Rosa* species.

Stage1

The eggs are oviposited in the bud on young leaves, before leaf expansion has occurred. They are attached to the leaf by a thin hair. The eggs become embedded into the leaf and as the leaf expands the cells around divide to form a small swelling on either side of the leaf with coarse hairs on the surface as shown in Figure 5.5 (A). Internal morphology (B) remains compact with small larval chambers and very little cortical parenchyma. The tissue sections (20μm) at this stage (C) and (D) show the larval chamber and the inner-gall cells at a higher magnification, respectively. There appears to be 3 layers of nutritive cells lining the chamber and in (D) they appear yellow in colour, suggesting they are lipid filled and the first layer is showing anticlinal division. The nuclei and nucleoli of the inner-cells do not appear as enlarged as those observed in the previous galls.

Stage 2

As the gall develops it increases in size and more hairs form on the surface, which are green or red, shown in Figure 5.5 (E). Internally (F) inner chambers, lined with nutritive tissue, house each larva and there is little parenchyma forming the outer gall. The tissue sections (G) and (H) show the chamber has increased in size and no distinct layers of nutritve cells can be observed (H).

Stage 3

The gall expands and the chamber also continues to increase in size, as shown in Figure 5.6 (A). The distinct inner-nutritive cells are not visible as in *B.pallida*, however, the cells have increased in size from stage one, but the two types of nutritive cells are not clearly distinguishable (B).

Stage 4

The gall begins to go brown and dry out as shown in Figure 5.6 (C). Internally the sclerenchyma capsules are all that remain once the tissue has been grazed and the larva are ready to pupate (Figure 5.6 (D)).

5.3.5 Andricus quercuscalicis development

A.quercuscalicis has two generations each year, as discussed in chapter 4, and the monolocular acorn gall was chosen for this investigation due to the abundance. Less is known about the development of *A.quercuscalicis* compared to the more studied galls like *B.pallida.*

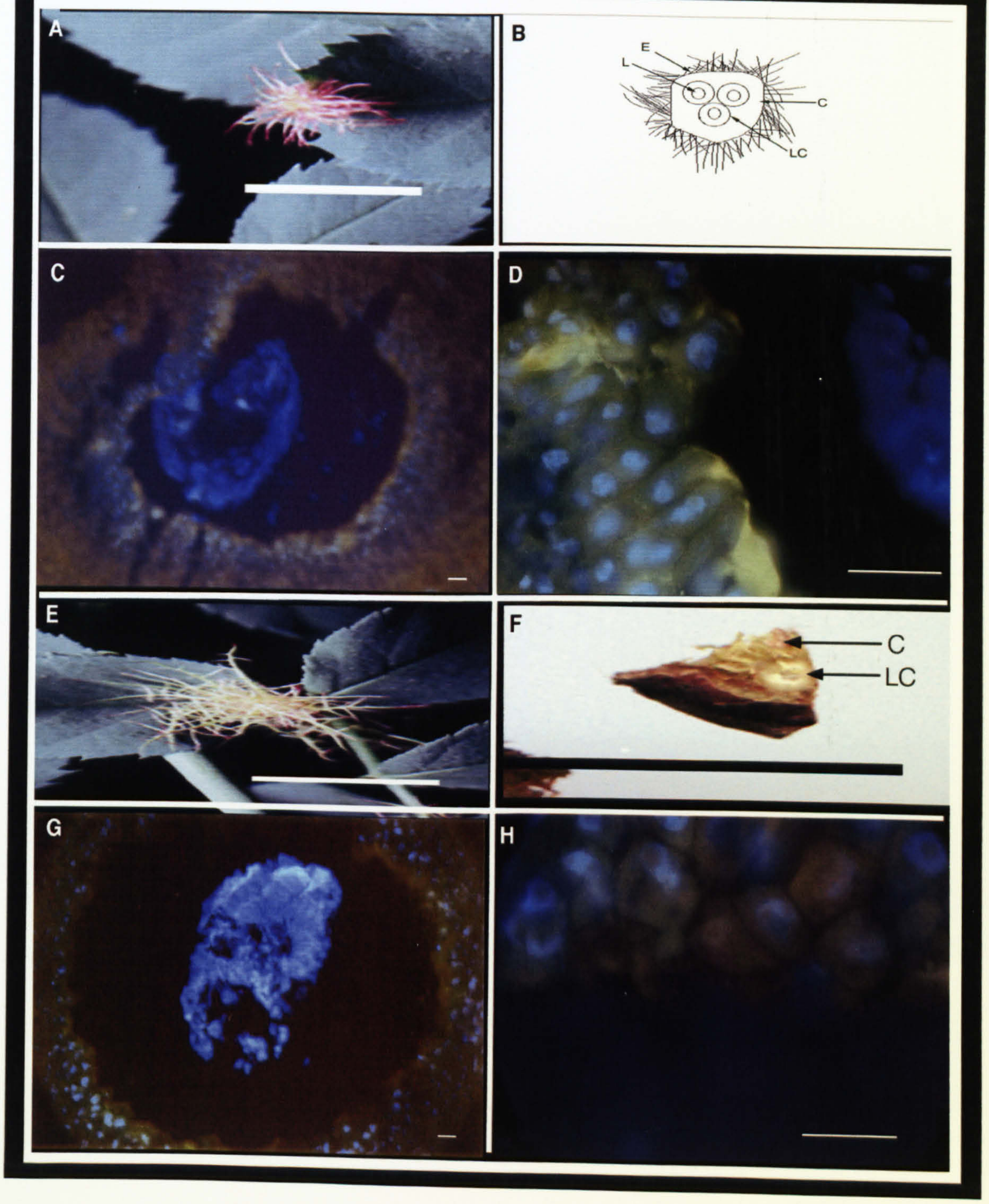


Figure 5.7 *D.rosae* at stage 1 and stage 2 of development. (A) External gall morphology at stage 1. (B) Line drawing of internal gall morphology at stage 1. (C) Tissue section of larval chamber at stage 1. (D) Tissue section of inner-gall cells at stage 1 stained with DAPI. (E) External gall morphology at stage 2. (F) Internal gall morphology at stage 2. (G) Tissue section of larval chamber at stage 2 stained with DAPI. (H) Tissue section of inner-gall cells at stage 2 stained with DAPI. LC = Larval chamber, C = Cortex, E = epdermis
A, B, E, F scale bar = 1cm. C, D, G, H scale bar = 50μm

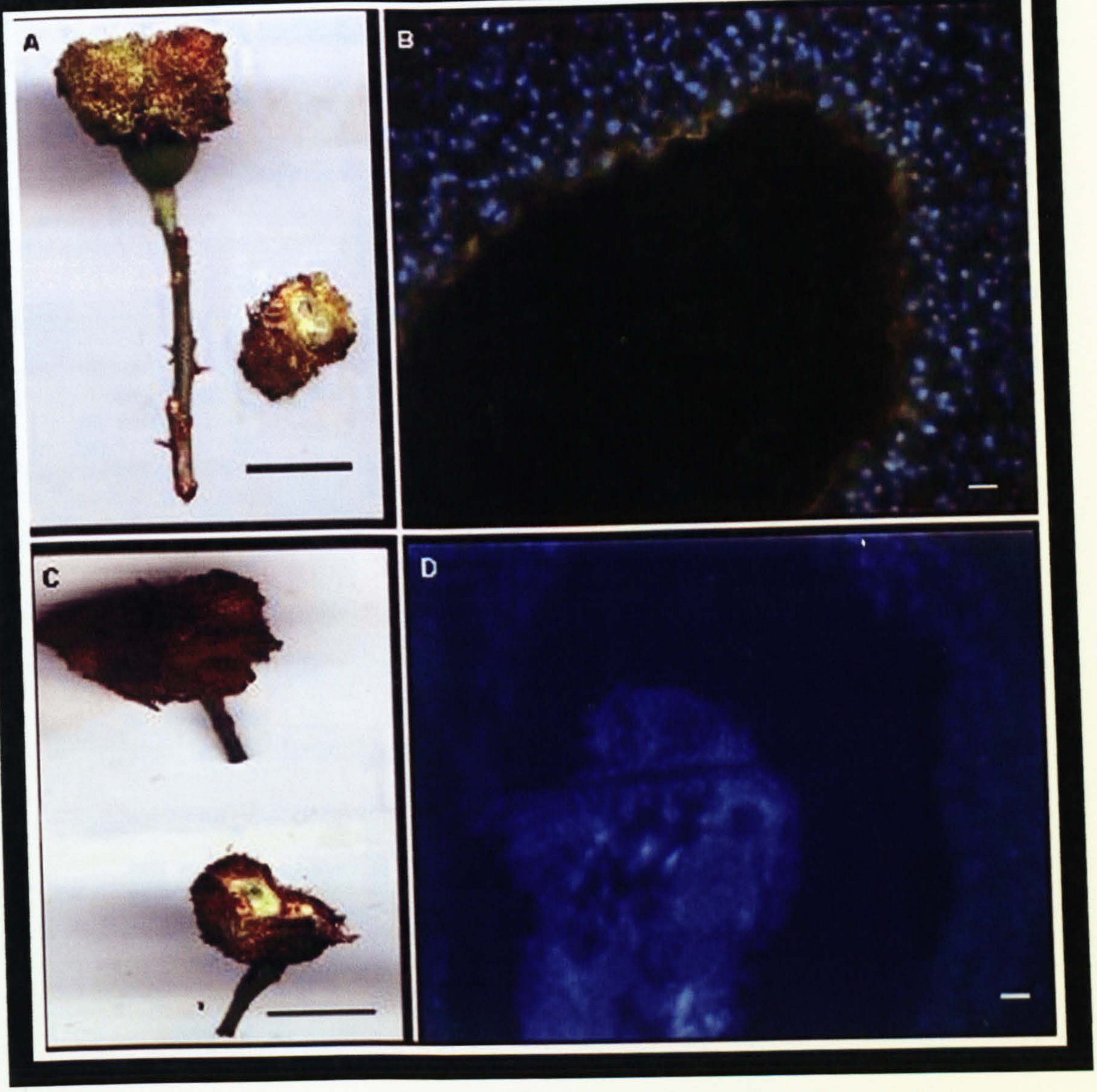


Figure 5.8 *D.rosae* at stage 3 and stage 4 of development. (A) External and internal gall morphology at stage 3. (B) Tissue section of larval chamber at stage 3 stained with DAPI. (C) External and internal gall morphology at stage 4. (D) Tissue section of the larval chamber and larvae at stage 4 stained with DAPI.
A, C scale bar = 1cm. B, D scale bar = 50μm

Stage 1

The gall forms at the base of the acorn between the acorn and the cup and when there is just a single gall, it forms at the side of the acorn, however, it can enclose the whole acorn if there are multiple galls on a single acorn. The presence of a gall first becomes apparent when the outer cortex pushes above the acorn cup. At this point we were able to take our first samples. The external (A) and internal (B) morphology and tissue sections (15-20μm) of the gall at this stage can be seen in Figure 5.9. Although the outer cortex has formed the gall is small and the inner chamber is small. This gall and *A.fecundator* demonstrate an interesting variation to *B.pallida, D.rosae, N.quercusbaccarum* and *C.quercusfolii.* The section in Figure 5.9 (C) shows that at this early stage a structure encapsulates the young larva. There is no layer of inner-gall tissue around this, only sclerenchymatic support around the structure. Lining the structure are 2-5 layers of cells, which appear to be lipid-filled and the first layer appears to have enlarged nuclei.

Stage 2

The gall increases in size as can be seen in Figure 5.9 (D) and (E). The chamber size increases due to the formation of a thick layer of soft nutritive tissue around the structure encapsulting the larva. The sections of the chamber at this stage in Figure 5.9 show the structure (F) and the inner-gall tissue surrounding this in (H). These inner-gall cells are much smaller than those lining the structure, and the other nutritive cells seen in the previous galls. These smaller cells do not appear to be lipid filled or have enlarged nuclei. The structure surrounding the larvae is possibly a cambium layer, forming enlarged nutritive cells inwards and smaller nutritive cells outwards, however, it appears to be a

more separate, egg-like-structure protecting and nurishing the larva. It is not seen in the previous galls, which form larger nutritve cells, suggesting it is a mechanism used to ensure the young larvae obtain sufficient nutrients until they are able to graze the smaller cells. The cells lining the egg-like structure can be seen in (G) and the first layer immediately surrounding the larva are enlarged and show anticlinal division and the layers behind this are smaller showing periclinal division.

Stage 3

The egg-like-structure breaks down and cells on one side of the larva appear to lyse and a cavity is formed. Remnants of the egg-like-structure can be seen under the larva, perhaps providing support for the developing larva. External (A) and internal (B) morphology and tissue sections of the gall at this stage can be seen in Figure 5.10. The larva is rapidly increasing in size and fills the cavity formed on one side of it. The tissue section of the chamber shows that, at this stage, only the smaller cells remain encapsulating the larva and they appear to be more tightly packed around the remains of the egg-like-structure. The inner-gall cells at higher magnification (D) shows there does not seem to be different inner and parenchymatic nutritive cells as in *B.pallida, N.quercusbaccarum* and *C.quercusfolii.* They appear the same size throughout the whole chamber.

Stage 4

A hard capsule of sclerenchyma surrounds the larva and the nutritive tissue and as the larva continues to develop it grazes the tissue until only the sclerenchyma remains. Figure 5.10 shows the external (E) and internal (F) morphology and tissue section (G) of the gall at this stage.

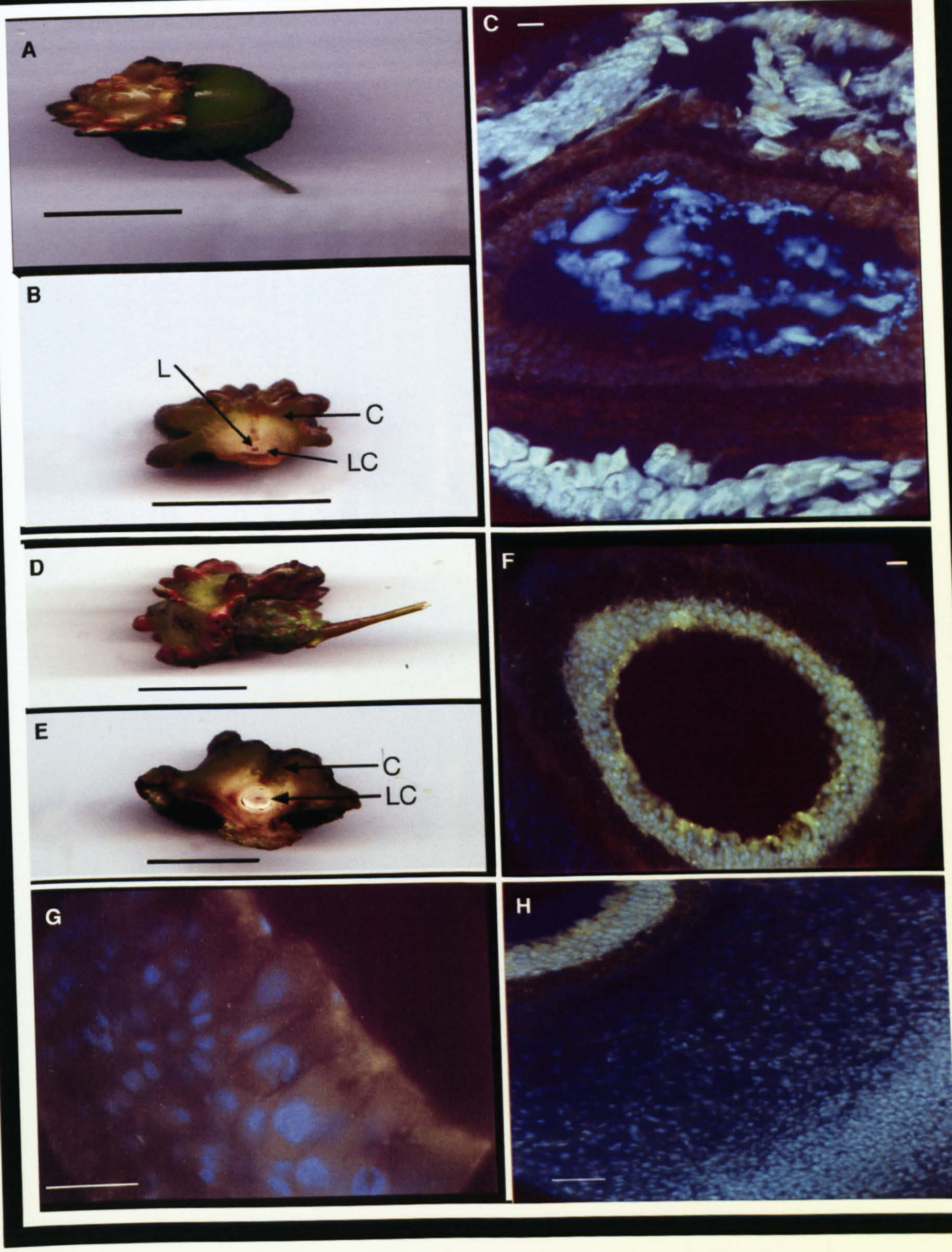


Figure 5.10 *A.quercuscalicis* at stage 3 and stage 4 of development. (A) External gall morphology at stage 3. (B) Internal gall morphology at stage 3. (C) Tissue section of larval chamber at stage 3 stained with DAPI. (D) Tissue section of inner-gall cells at stage 3 stained with DAPI. (E) External gall morphology at stage 4. (F) Internal gall morphology at stage 4. (F) Tissue section of larval chamber at stage 4 stained with DAPI.

A, B, E, F scale bar = 1cm. C, D, G, scale bar = 50μm

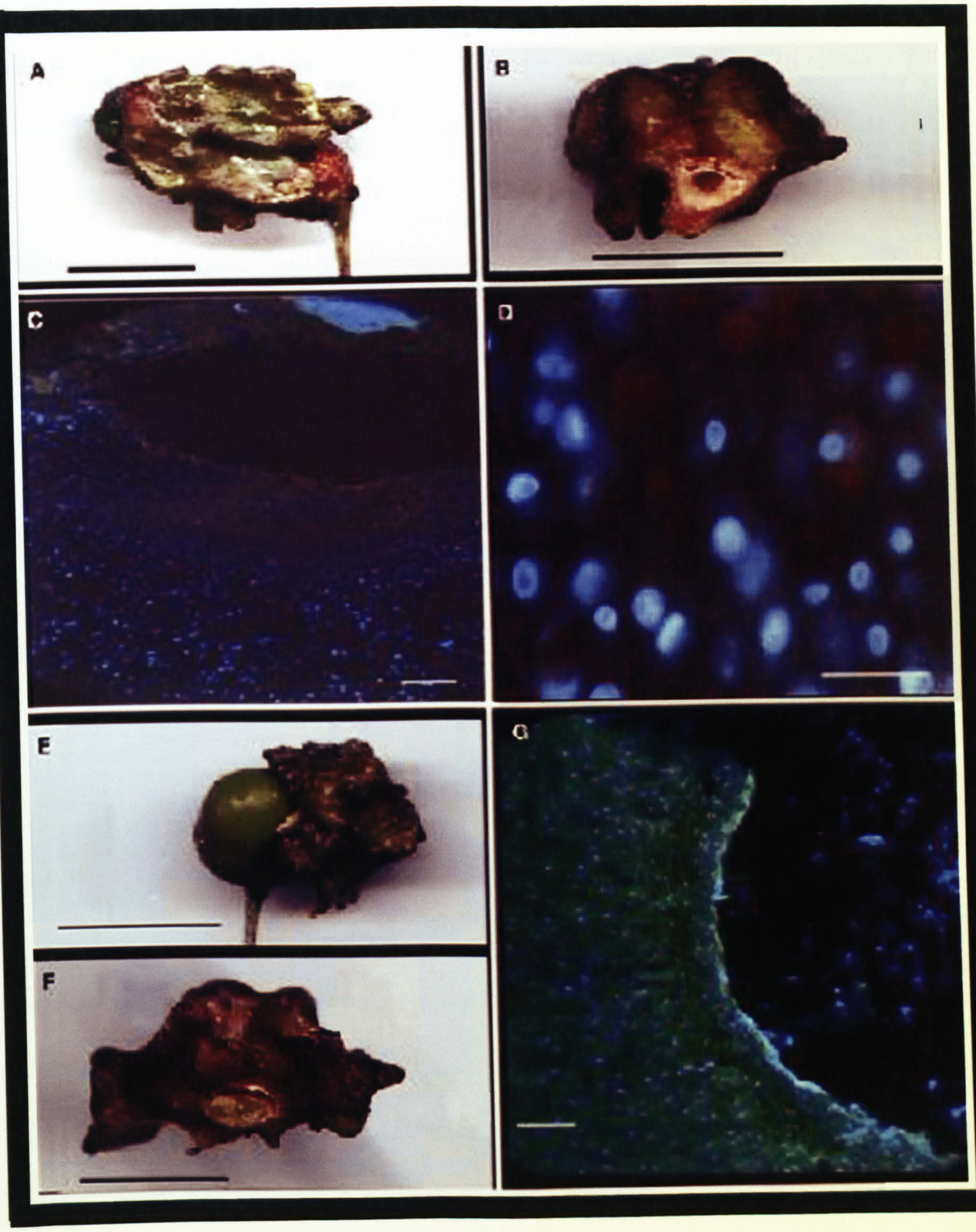


Figure 5.9 *A.quercuscalicis* at stage 1 and stage 2 of development. (A) External gall morphology at stage 1. (B) Internal gall morphology at stage 1. (C) Tissue section of larval chamber at stage 1 stained with DAPI. (D) External gall morphology at stage 2. (E) Internal gall morphology at stage 2. (F) Tissue section of the larval chamber at stage 2 stained with DAPI. (G) Tissue section of inner-gall cells at stage 2 stained with DAPI. (H) Tissue section of the outer part of the chamber at stage 2 stained with DAPI. LC = Larval chamber, C = Cortex
A,B,D,E scale bar = 1cm C,F,G,H scale bar = 50μm

5.3.6 Andricus fecundator development

This species has two generations a year, as dicussed in chapter, 4 and the autumn generation monolocular bud gall on *Q.robur* was used for our investigation.

Stage1

The eggs are oviposited in the buds of *Q.robur* and Figure 5.11 shows the external (A) and internal (B) morphologies, even at this stage the small pine cone structure is visible. The outer part of the gall is different to previous galls discussed as it is formed from enlarged and modified bud scales in which the egg was laid. The whole outer gall consists of layers of scales and there is no soft cortical parenchyma. The tissue section of the chamber (C) shows an egg-like structure encapsulating the larva, lined with 3-4 layers of cells. There is no inner-gall tissue on the outside of the structure and the outer-gall is small, as in *A.quercuscalicis*.

Stage 2

The gall increases in size as does the chamber, shown in Figure 5.11 (D) and (E). As in *A.quercuscalicis*, the inner-gall tissue forms and a distinct chamber buried within the scales is formed, as can be seen in the tissue section in Figure 5.9 (F). The inner-gall tissue cells appear smaller than those seen in *B.pallida* and as can be seen in tissue section (G) showing the nutritive cells at a higher magnification.

Stage3

The larva grazes on the inner-gall tissue and continues to develop. Little change occurs to the external size or morphology, Figure 5.12 (A). Internally (B) a

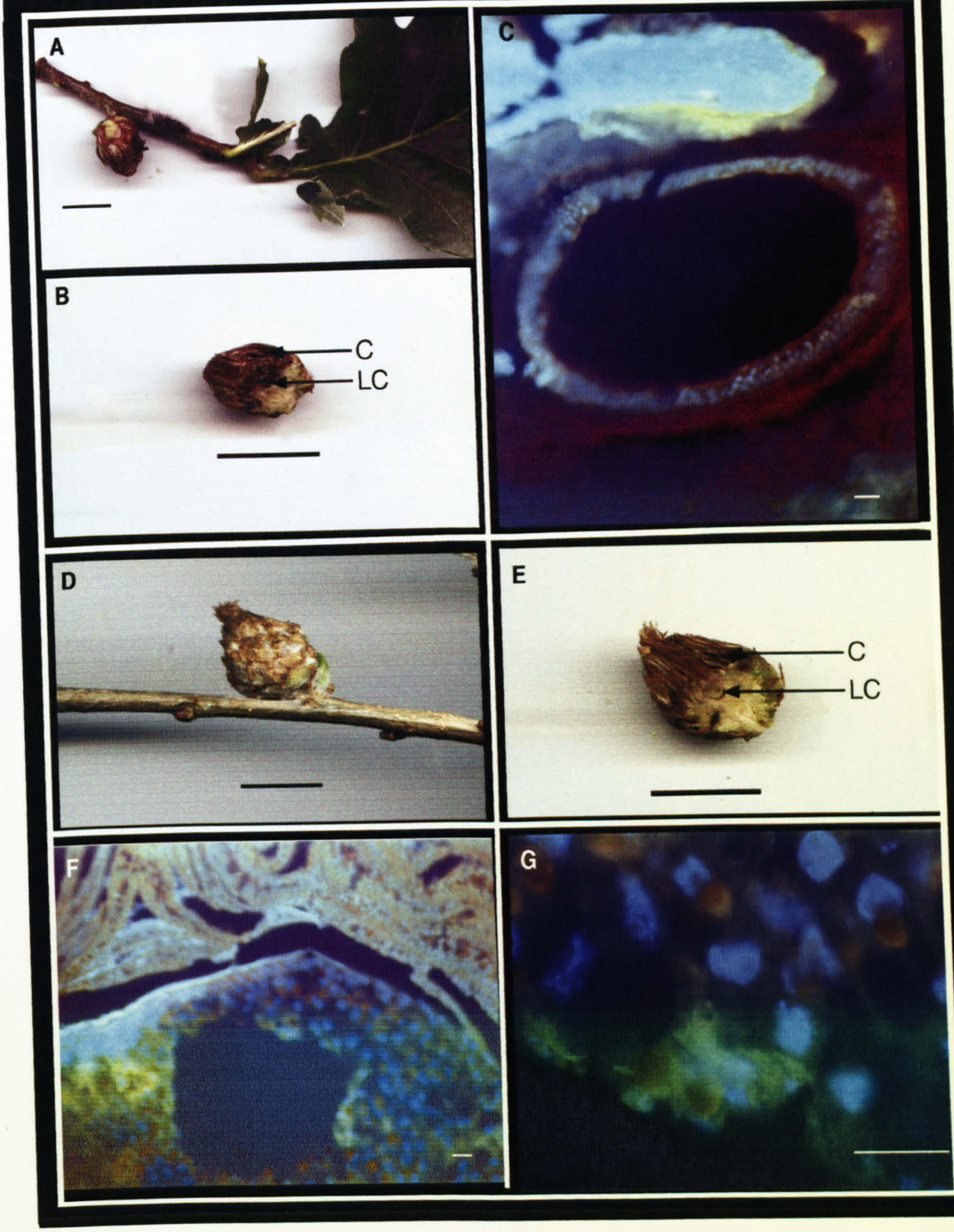


Figure 5.11 *A.fecundator* at stage 1 and stage 2 of development. (A) External gall morphology at stage 1. (B) Internal gall morphology at stage 1. (C) Tissue section of larval chamber at stage 1 stained with DAPI. (D) External gall morphology at stage 2. (E) Internal gall morphology at stage 2. (F) Tissue section of the larval chamber at stage 2 stained with DAPI. (G) Tissue section of inner-gall cells at stage 2 stained with DAPI. LC = Larval chamber, C = Cortex
A, B, D, E scale bar = 1cm. C, F, G scale bar = 50μm

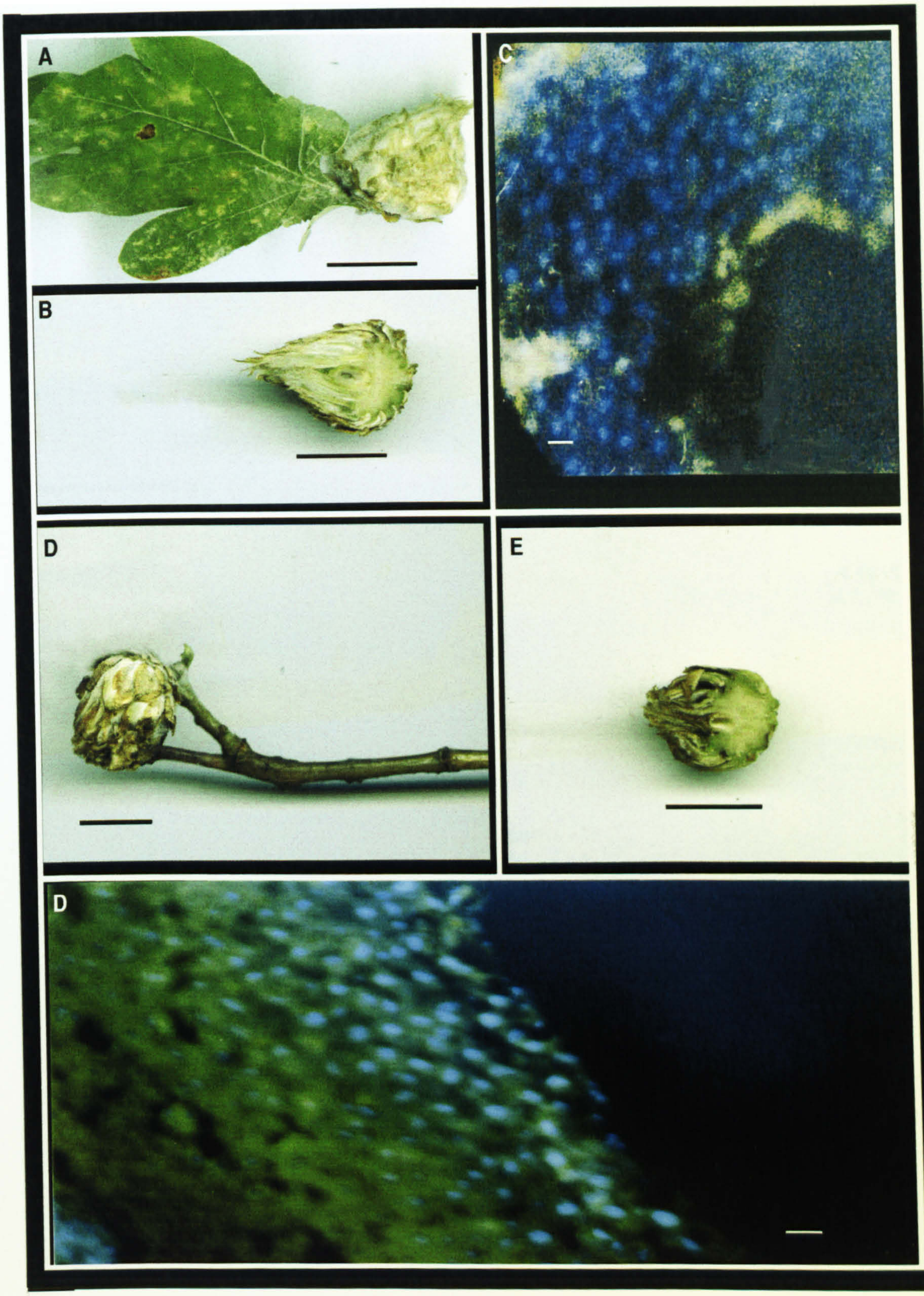


Figure 5.12 *A.fecundator* at stage 3 and stage 4 of development. (A) External gall morphology at stage 3. (B) Internal gall morphology at stage 3. (C) Tissue section of larval chamber at stage 3 stained with DAPI. (D) External gall morphology at stage 4. (E) Internal gall morphology at stage 4. (F) Tissue section of larval chamber at stage 4 stained with DAPI.
A, B, D, E scale bar = 1cm. C, D scale bar = 50μm

sclerenchyma layer forms around the chambers, forming a more cylindrical shaped chamber, compared to the egg shaped chamber in *A.quercuscalicis*. The tissue section (C) shows the inner-gall tissue consists of small, densely packed cells and does not have the enlarged cells seen in *B.pallida*.

Stage4

The larvae graze all the inner-gall tissue and are surrounded only by the sclerenchyma layer, ready to pupate. The chamber is pushed up through the centre of the gall and expelled, where the larva pupates and emerges. The external (D) and internal (E) gall morphologies and tissue section (F) at this stage are shown in Figure 5.12.

5.3.7 Similarities and differences between cynipid species throughout gall development

From the tissue sections, there appears to be two main patterns of development. One which involves the presence of an egg-like- structure, seen in A. *quercuscalicis* and *A.fecundator*, in which the larva is encapsulated in the early stages of gall development and is not observed in *B.pallida, N.quercusbaccarum, C.quercusfolii* and *D.rosae*. The cells lining this structure are probably secretory cells used to supply nutrients to the larva in the early stages of development, before the larval mouthparts have formed. Once the larva develops strong mandibles, these can be used to graze the smaller nutritive tissue, after the breakdown of the egg-like -structure. These nutritive cells in the *Andricus* galls are much smaller than those seen in *B.pallida, N.quercusbaccarum, C.quercusfolii* and *D.rosae*, which show enlarged cells immediately surrounding the larva, although *D.rosae* to a lesser extent. Despite the differences between the *Andricus* galls and the others used, there are also important similarities. Both form the

characteristic enlarged, lipid-filled cells. *Andricus* galls form these early on in their development and rely on smaller nutritive cells for the later stages of development. *B.pallida* and galls showing similar patterns of development do not form these as early, however, once formed rely on the enlarged nutritive cells for most of the development. All the larvae are clearly inducing the formation of highly active, probably secretory cells, capable of producing sufficient amounts of the correct nutrients for the larva. This cell type, therefore, appears important for all cynipid galls and the distinct characteristics could help determine a signal responsible for such an essential cell type and possibly be used as markers in the bioassay.

5.4 Putative BCCP distribution throughout gall development.

The expression of the putative BCCP can be detected by western blotting of inner-gall protein extracts, throughout all the defined stages of gall development in all the galls tested (as shown in chapter 4). To investigate the spatial distribution of the protein expression in the gall, immunohistochemistry was used, hybridising tissue sections of the galls with Cy3-conjugated streptavidin. This flurochrome labelled streptavidin, should bind to the biotinylated protein and when visualised under fluorescent microscope the hybridisation can be detected by the red fluorescing Cy-3.

5.4.1 BCCP distribution in Biorhiza pallida

From stage 1 putative BCCP can be detected in the inner-gall tissue, as shown by the westerns. The tissue sections of the chamber in Figure 5.13 A-D show the cells immediately surrounding the larva in stage 1 are expressing the biotinylated protein. By stage 2 the layers of cells expressing BCCP has increased as shown in Figure 5.13

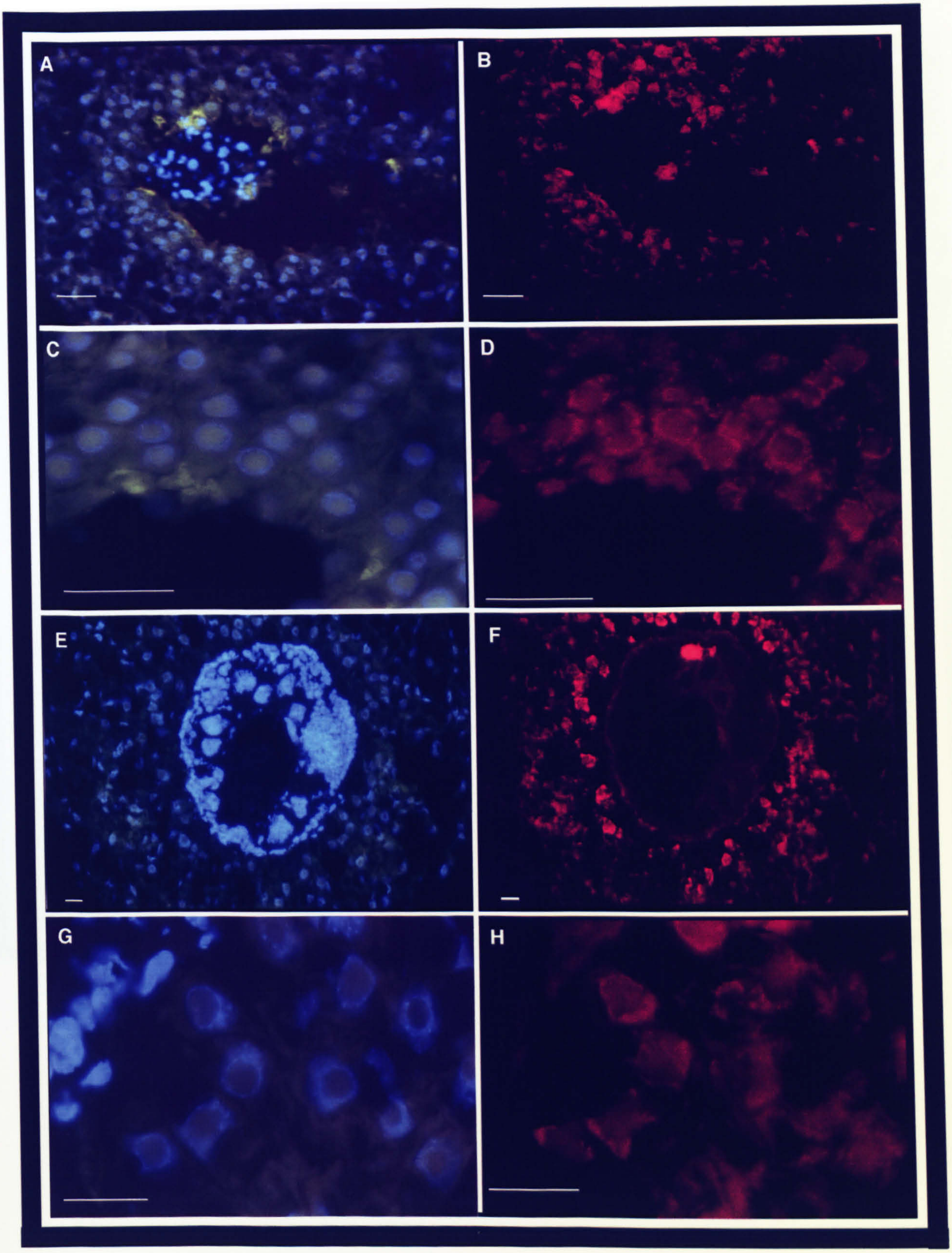


Figure 5.13 Immunohistochemical localisation of putative BCCP in *B.pallida* at stages 1 and 2. A) Tissue section of gall chamber at stage 1 stained with DAPI. B) Tissue section of gall chamber at stage 1 hybridised with Cy-3 conjugted streptavidin (red) C) Tissue section of inner gall cells stained with DAPI. D) Tissue section of inner gall cells at stage 1 stained with DAPI hybridised with Cy-3 conjugted streptavidin. E) Tissue section of gall chamber at stage 2 stained with DAPI F) Tissue section of gall chamber at stage 2 hybridised with Cy-3 conjugted streptavidin (red) G) Tissue section of inner gall cells at stage 2 stained with DAPI. H) Tissue section of inner gall cells at stage 2 with Cy-3 conjugted streptavidin.
Scale bar = 50μm

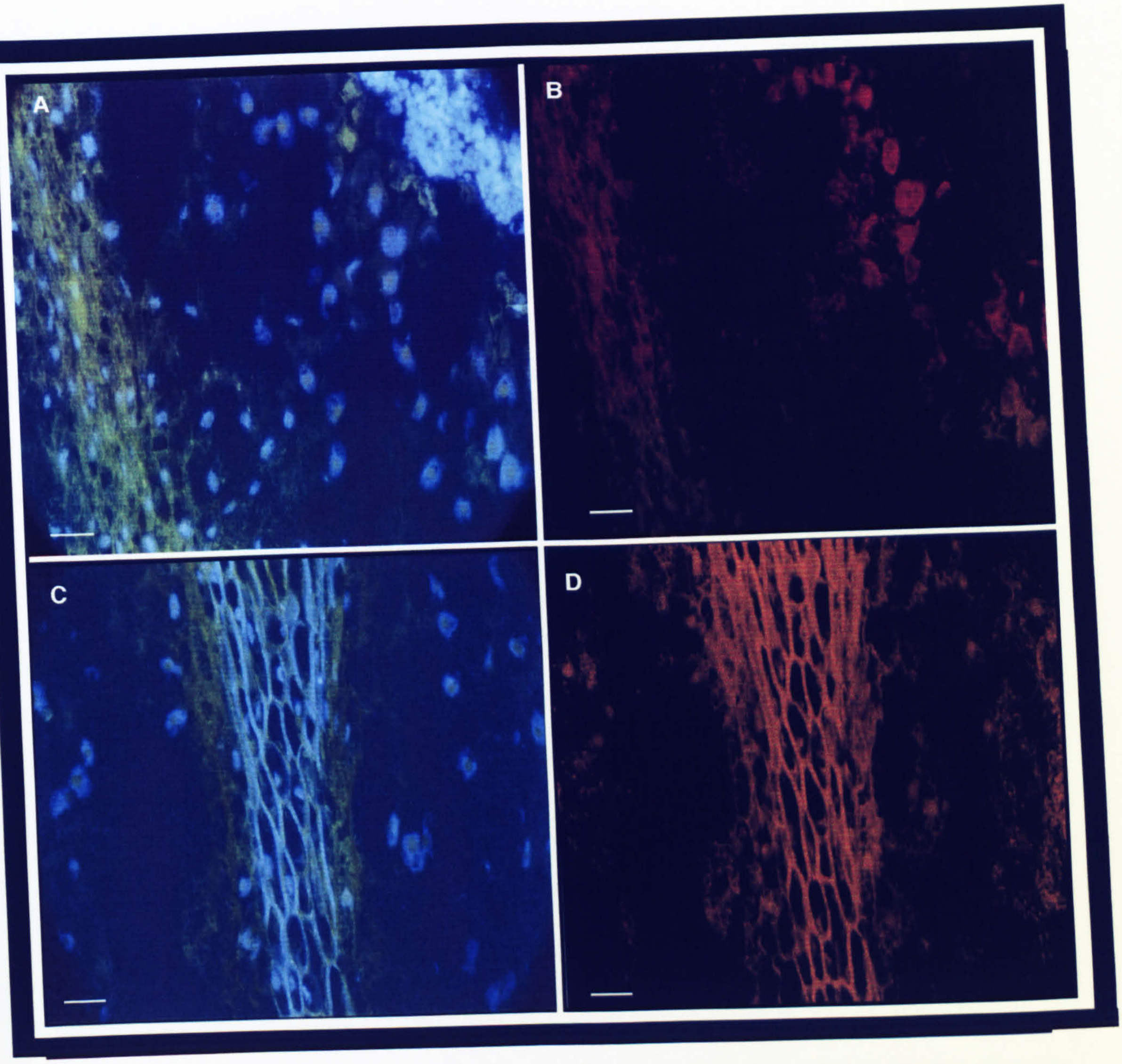


Figure 5.14 Immunohistochemical localisation of putative BCCP in *B.pallida* at stage 4. A) Tissue section of gall chamber at stage 4 stained with DAPI B) Tissue section of gall chamber at stage 4 hybridised with Cy-3 conjugated streptavidin (red) C) Tissue section of inner gall cells stage 4 stained with DAPI. D) Tissue section of inner gall cells at stage 4 stained with DAPI hybridised with Cy-3 conjugated streptavidin.
Scale bar = 50μm

E-H. It is possible to see a decreasing gradient of expression away from the larva. In the outermost part of the chamber, there is no expression. By stage 4 there is only a thin layer of inner-gall tissue remaining and BCCP expression is detected in these cells shown in Figure 5.14 A-D. A non-specific signal can also be seen against the schlerenchymatic larval capsule. BCCP is nuclear encoded and resides in the chloroplast or plastids, where lipid synthesis occurs. The biotinylated protein detected here appears to be concentrated around the nucleus and may be located in plastids immediately surrounding the nucleus. It is possible additional biotinylated proteins, which are not BCCP are being detected, however, only a 35kDa biotinylated protein was detected using western analysis on protein extracts. To confirm it is BCCP being detected a specific antibody would need to be used. An antibody to *Arabidopsis* BCCP has been obtained from Prof. Nikolau from Iowa State University, however, this was not able to be used within the time of the project.

5.4.2 BCCP distribution in Neuroterus quercusbaccarum

Like *B.pallida* the expression of BCCP can be detected throughout development by western blotting. The tissue (15μm) sections shown in Figure 5.15 A-D reveal that the distribution is concentrated mainly in the inner-nutritive cells immediately surrounding the larva, shown in (D). As with *B.pallida*, a decreasing gradient away from the larva is observed. Again, the signal is concentrated around the nucleus.

5.4.3 BCCP distribution in Cynips quercusfolii

C.quercusfolii also shows the expression of putative BCCP throughout development by western blotting. The tissue sections in Figure 5.16 A-D show that the distribution is concentrated in the first few layers of cells immediately surrounding the larva. The

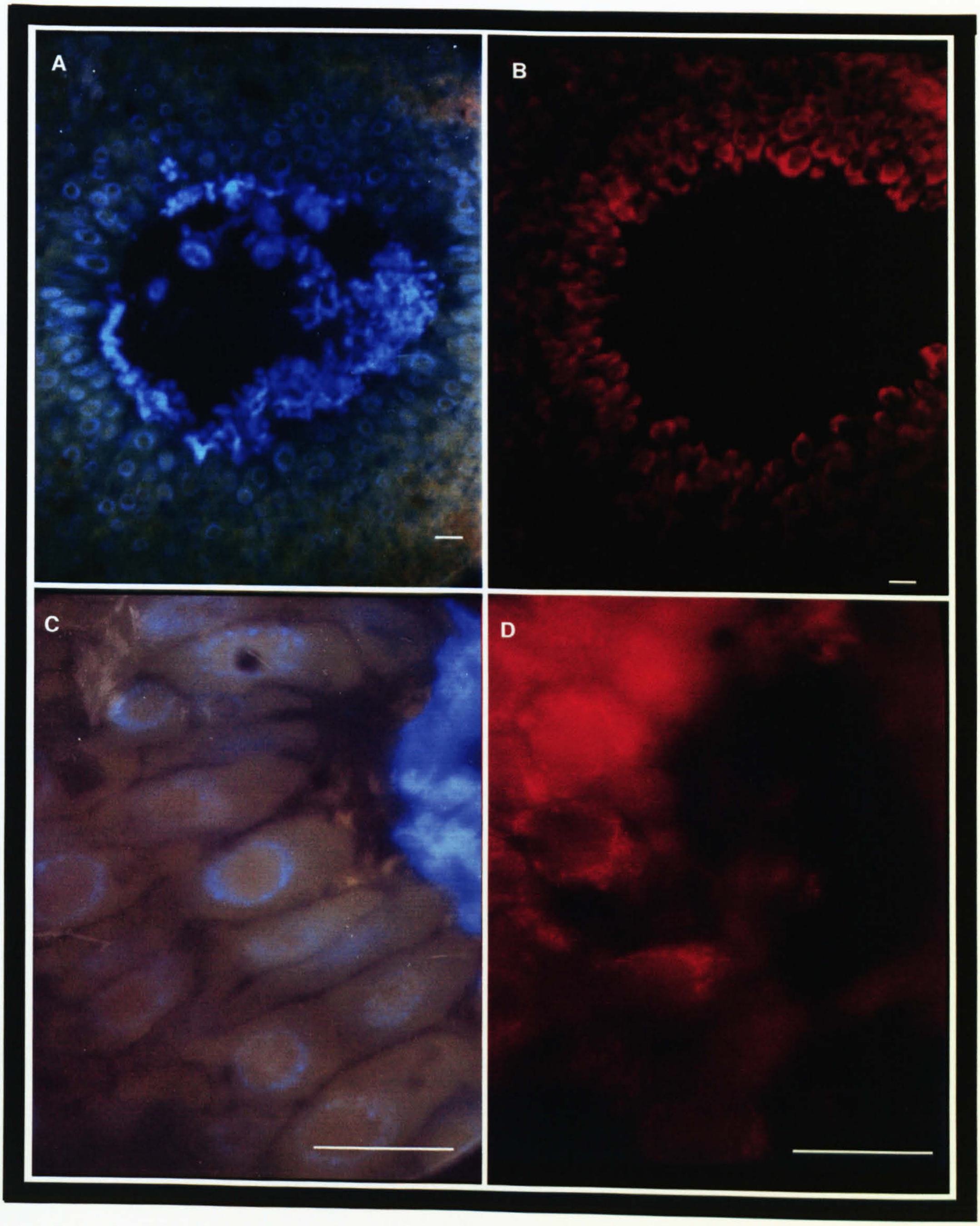


Figure 5.15 Immunohistochemical localisation of putative BCCP in *N.quercuscalicis* at stage 2. A) Tissue section of gall chamber at stage 2 stained with DAPI B) Tissue section of gall chamber at stage 2 hybridised with Cy-3 conjugated streptavidin (red) C) Tissue section of inner gall cells at stage 3 stained with DAPI. D) Tissue section of inner gall cells at stage 3 hybridised with Cy-3 conjugated streptavidin.
Scale bar = 50μm

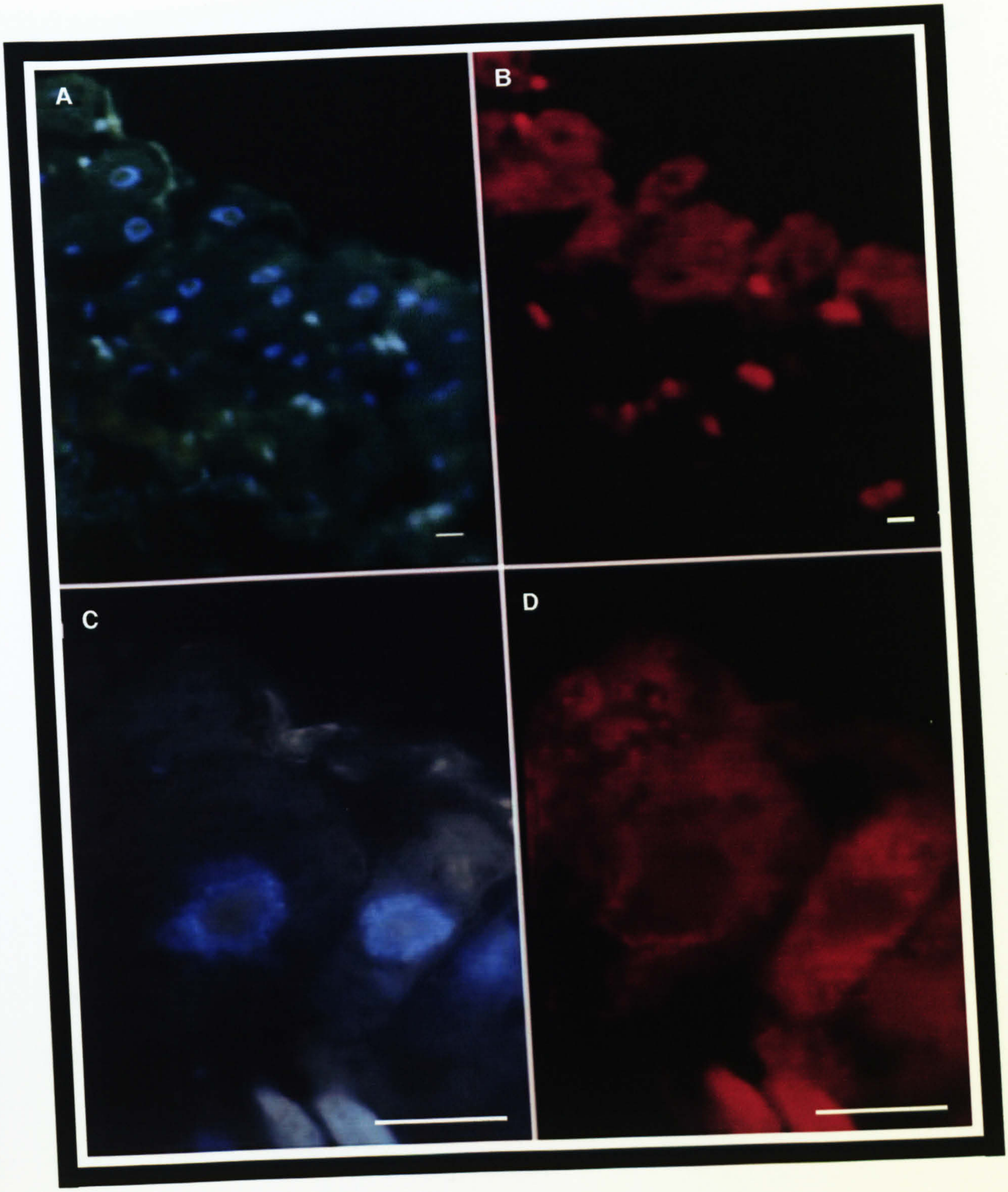


Figure 5.16 Immunohistochemical localisation of putative BCCP in *C.quercusfolii* at stage 2. A) Tissue section of gall chamber at stage 1 stained with DAPI B) Tissue section of gall chamber at stage 1 hybridised with Cy-3 conjugated streptavidin (red) C) Tissue section of inner gall cells at stage 2 stained with DAPI. D) Tissue section of inner gall cells at stage 2 hybridised with Cy-3 conjugated streptavidin.
Scale bar = 50μm

decreasing gradient away from the larva is much steeper than observed in *B.pallida* and no expression is seen in the cells in the outer part of the chamber.

5.4.4 BCCP distribution in Andricus quercuscalicis

From the westerns, the expression of BCCP is expressed in all stages of *A.quercuscalicis* development. The distribution of the biotinylated protein can be seen in Figure 5.17 A-D. The tissue sections (15μm) of stage 2 (A and B) show that the enlarged cells lining the egg-like-structure are expressing the BCCP. As the gall develops the small cells which form around the egg-like-structure show only a little signal (C and D). When the egg-like structure disappears, there still appears to be expression from the remaining cells, however, if these are necrosed then this may be a false signal. The cells surrounding this show little expression throughout the development, which corresponds to the decrease in signal observed in the western analysis. In the western blot, the inner-gall extract from stage 3 had a significantly weaker signal from stage 2. The signal decreased further in stage 4, suggesting BCCP expression is declining in the inner-gall tissue as the gall develops. The tissue sections confirm that the biotinylated protein is present mainly in the cells lining the egg-like–structure and when these disappear the protein is only present at low levels in the inner-gall tissue.

5.4.5 BCCP distribution in Andricus fecundator

The expression of the biotinylated protein is detected throughout development of *A.fecudator* by western blotting. The tissue sections in Figure 5.18 A-G reveal the distribution follows that observed in *A.quercuscalicis*. The cells lining the egg-like-structure show high levels of expression (A and B), and when the small cells form

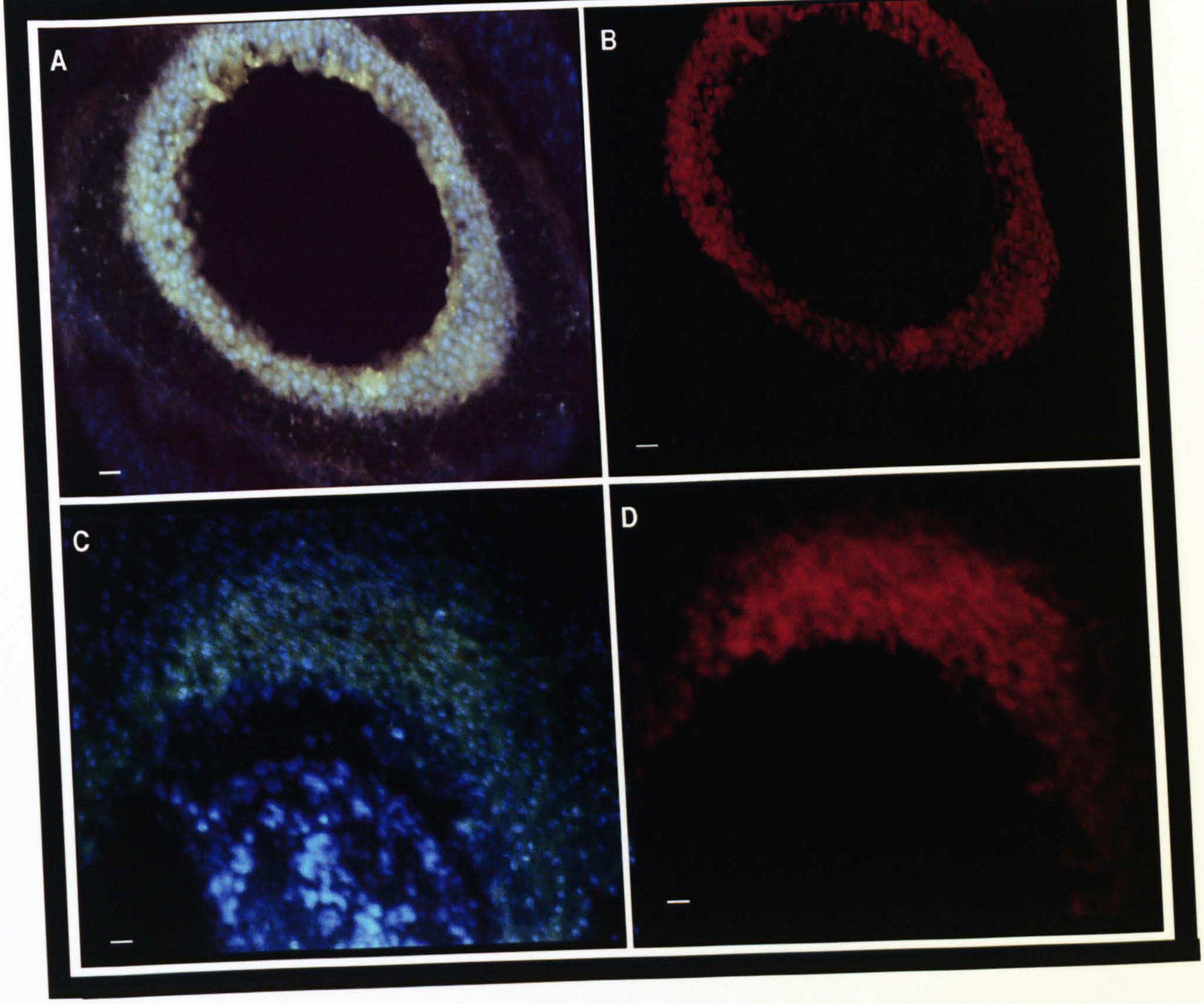


Figure 5.17 Immunohistochemical localisation of putative BCCP in *A.quercuscalicis* at stage 2 and 3. A) Tissue section of gall chamber at stage 2 stained with DAPI B) Tissue section of gall chamber at stage 2 hybridised with Cy-3 conjugated streptavidin (red) C) Tissue section of gall chamber at stage 3 stained with DAPI. D) Tissue section of gall chamber at stage 3 hybridised with Cy-3 conjugated streptavidin.
Scale bar = 50μm

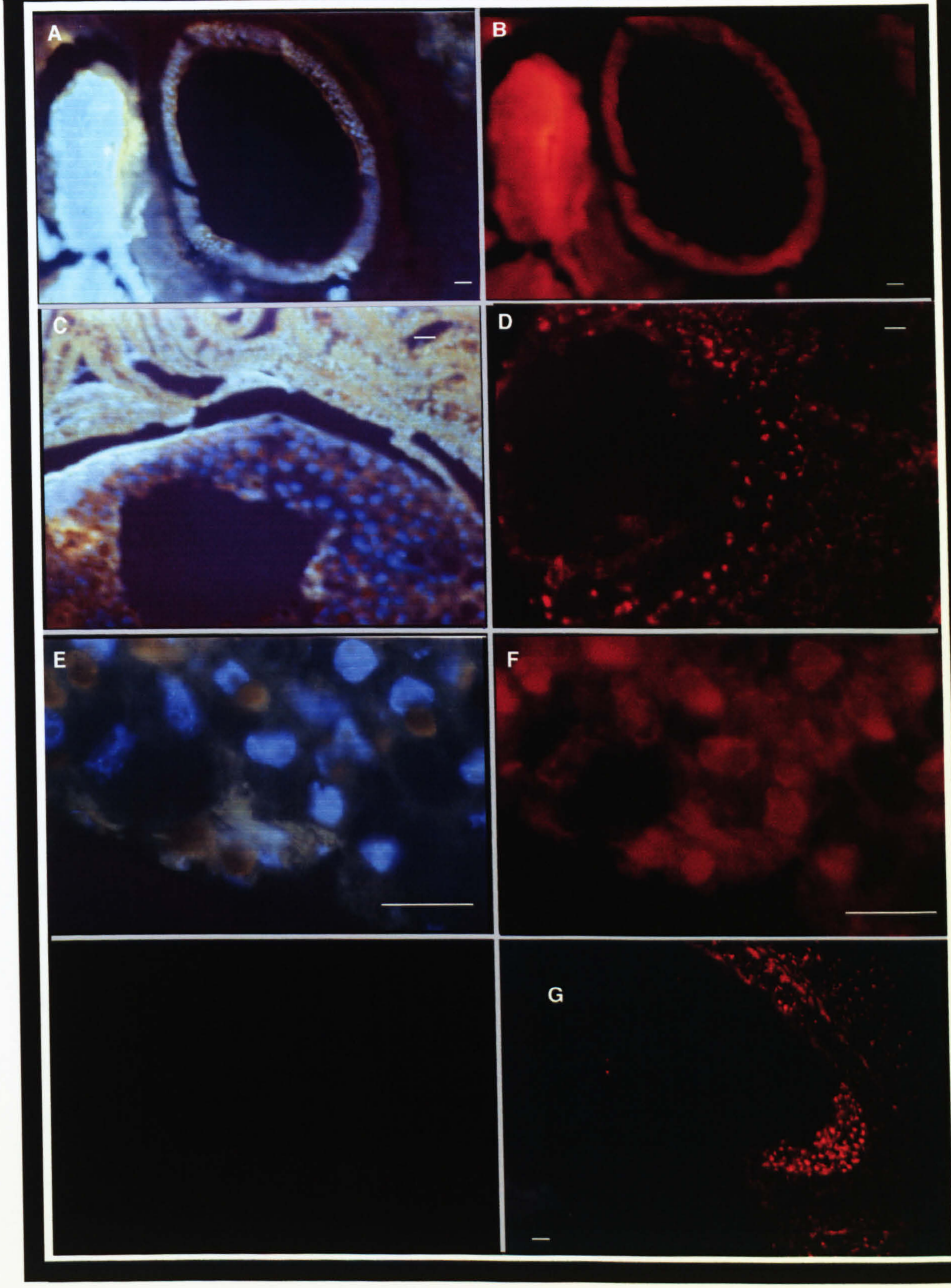


Figure 5.18 Immunohistochemical localisation putative BCCP in *A.fecundator* at stage 1, 2 and 4.
A) Tissue section of gall chamber at stage 1 stained with DAPI B) Tissue section of gall chamber at stage 1 hybridised with Cy-3 conjugated streptavidin (red) C) Tissue section of gall chamber at stage 2 stained with DAPI. D) Tissue section of gall chamber at stage 2 hybridised with Cy-3 conjugated streptavidin (red) E) Tissue section of inner gall cells at stage 4 stained with DAPI. F) Tissue section of inner gall cells at stage 4 hybridised with Cy-3 conjugated streptavidin. G)Tissue section of gall chamber at stage 4 hybridised with Cy-3 conjugated streptavidin.
Scale bar = 50µm

around this they show a lower level of expression. When the egg-like-structure has disappeared, the inner-gall cells appear to express higher levels than those observed in *A.quercuscalicis* (C, D, E and F). This corresponds to the intensity of signal observed in the western. In stage 3 the intensity of the signal does not appear to decrease, as observed in *A.quercuscalicis*, and higher expression in the remains of the inner-gall tissue can be seen in the tissue section (G).

5.5 Chromosome alterations

5.5.1 Protoplast analysis of inner-gall nutritive cells

The most abundant gall available, which demonstrated clearly endoreduplication of the nuclei from the tissue sections, was *B.pallida*. To investigate this further, protoplasts of inner-gall tissue were prepared using a variety of enzymes to break down the cell wall and digest the cytoplasm. Inner-gall tissues from all stages were used and analysed by staining with DAPI and visualising under a fluorescent microscope. Figure 5.19 shows the nutritive cell nuclei at 3 stages of development and non-gall tissue nuclei from oak root tip cells. The oak root tip prophase nuclei, prepared by Dr Y. Lim, shows 24 chromosomes (A). Compared to this, the inner-gall cell nuclei appear enlarged at all the stages and the chromosome structure changes throughout development. In the early stages the thin thread like chromosomes can be seen, showing no clear signs of polytenisation (C). As the gall develops and many layers of the inner-nutritive cells form, the genome becomes polytene (E). The chromosome structure is changing and they now appear as a cable-like-structure, composed of many sister chromatids held together at the centromere. This is an incomplete nuclei and not all of the 24 chromosomes can be seen in (E). In (G) all 24 polytene chromosomes can clearly be seen and the chromatids fan out from the

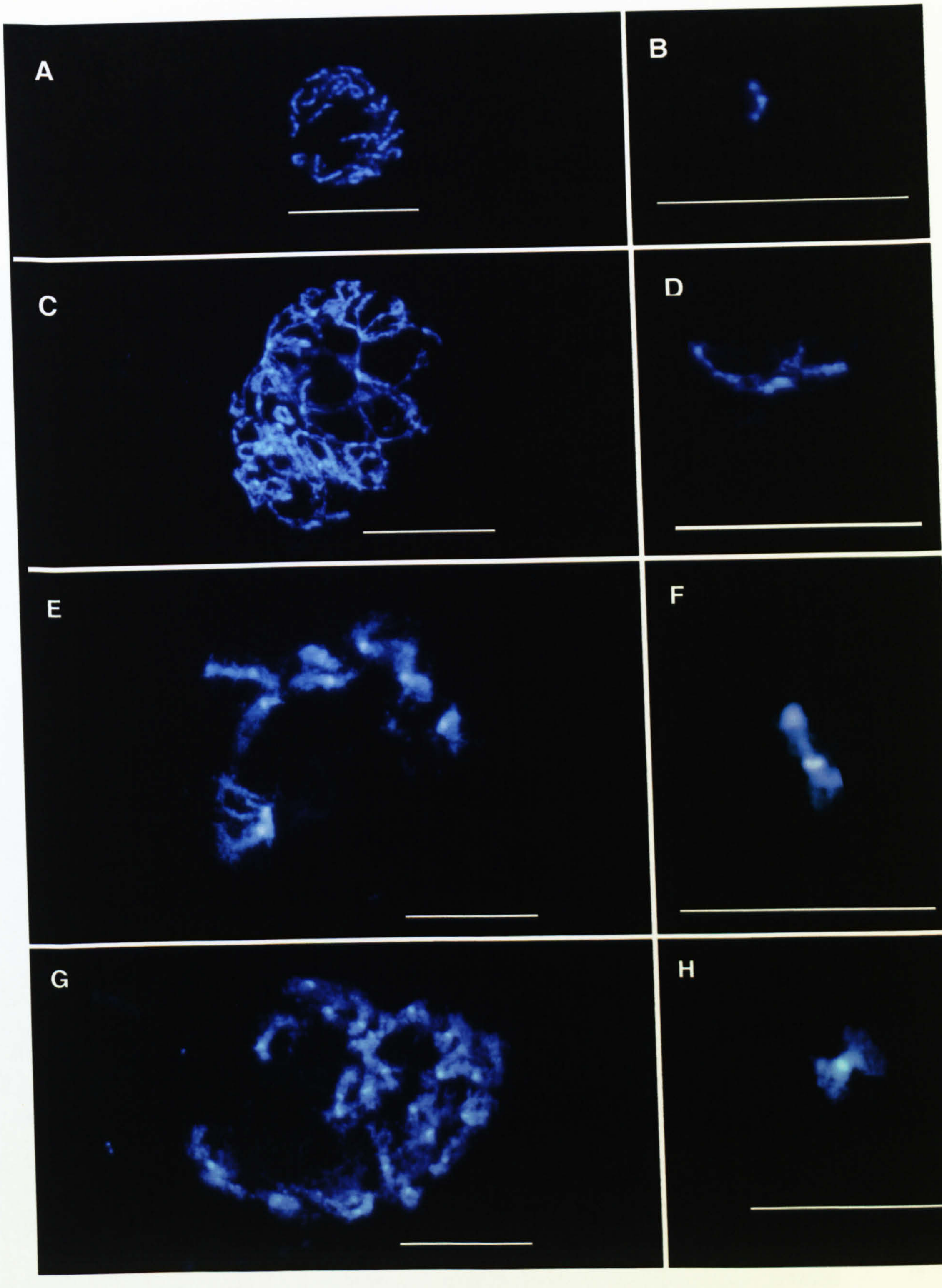


Figure 5.19 Nuclei of oak root tip and *B.pallida* inner-gall cells at different stages of development stained with DAPI. (A) *Q.robur* root tip nuclei in prophase. (B) Single root tip chromosome in prophase. (C) *B.pallida* stage 1 nuclei. (D) Single chromosome from *B.pallida* stage 1. (E) *B.pallida* stage 2 nuclei. (F)) Single chromosome from *B.pallida* stage 2 nuclei. (G) *B.pallida* stage 3 nuclei. (H) Single chromosome from *B.pallida* stage 3 nuclei.
Scale bar = 50μm

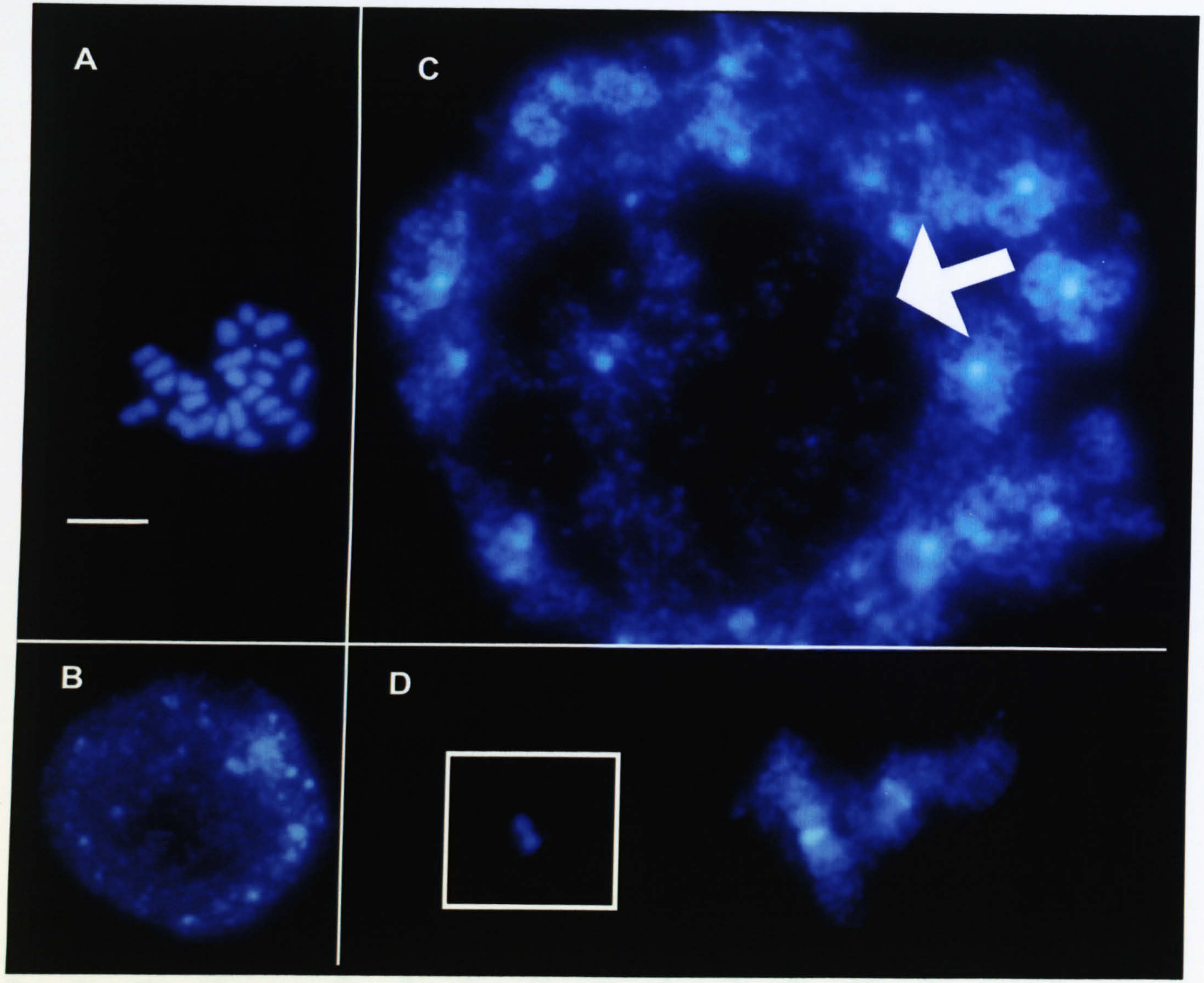


Figure 5.20 Polytene nuclei of oak root tip and *B.pallida* inner-gall tissue stained with DAPI. (A) *Q.robur* root tip nucleus in metaphase. (B) *Q.robur* root tip nucleus in interphase stained. (C) *B.pallida* polytene nucleus. (D) *B.pallida* polytene chromosome showing "bow-tie" arrangement. Scale bar = 50µm

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centromere, forming a characterisitic bow-tie shape (H). Figure 5.20 highlights the difference between non-gall and inner-gall nuclei. The increased nucleolus of the gall nuclei can also be seen in Fig.5.20 (C), enabling increased translation.

5.5.2 FISH on Biorhiza pallida inner-gall nuclei

Fluorescent *in-situ*-hybridisation using rDNA probe was used on the polytene nuclei to assess the state of polytenisation and if the rDNA loci were being reduplicated. It is possible that these are not reduplicated as in some instances of endoreduplication, only the euchromatin is reduplicated and the heterchromatic regions such as that around the centromere remain contracted and do not reduplicate. Inner-gall nutritive cell nuclei were prepared as above and spread and fixed onto chromic acid washed slides. To carry out *in-situ* hybridisation the slides were denatured and hybridised with digoxygenin-labelled rDNA probe (see section 2.4.4), these were washed and detected using a FITC-labelled-anti digoxygenin probe and examined using a fluorescent microscope. Figure 5.21 shows the results of the *in-situ* hybridisation probing for the rDNA loci. The root tip nuclei (A), prepared by Dr Y. Lim, shows 4 18s,5.8s,26s rDNA sites (green), 2 major and 2 minor. The 2 5s rDNA loci can be seen in red. (B) and (C) clearly show the increase in size of the loci in the gall polytene nuclei in the centromeric region. This suggests that heterochromatic regions are amplified as well as euchromatic regions. (D) and (E) show the DAPI stained polytene chromosomes (D) and amplification of the rDNA loci (E) on the individual chromatids, fanning out from the centromere, marked by an arrow in (D). In (E) there are nine visible loci and 5 of these appear to be at least double, suggesting the nuclei is at least 16n, however, it is likely to be greater than this.

5.6 Cell specific markers

Previous, unpublished work carried out in the laboratory by Karsten Schönrogge demonstrated the presence of AGPs in gall tissue and larva. This was shown by Yariv reagent and AGP antibodies obtained from Dr Keith Roberts. Dot blots of non-gall tissue extract, inner-gall tissue extract and larval extract were incubated with Yariv reagent and positive staining was shown in stem, leaf and inner-gall tissue extracts. JIM4, JIM13 and Mac207 antibodies against AGPs were used to confirm and further investigate the presence of AGPs. Mac207, which binds to a wide range of AGPs gave a positive signal with inner-gall tissue, larval extract, oak leaf and stem extracts. JIM4, which has a narrower range of AGPs, gave positive signals on inner-gall tissue and larval extracts. JIM13 did not give positive results. This demonstrates that AGPs are in inner-gall tissue and in addition to those normally expressed in oak tissue, detected by Mac207, there appears to be different ones in the inner-gall and larva, as detected by JIM4.

5.6.1 AGP expression throughout gall tissue

To complement this work and to investigate the distribution of the AGPs detected by JIM4 and Mac207 I used tissue prints of *B.pallida* and *N.quercusbaccarum*. The galls were sliced and stamped onto HybondC membrane, which was blocked and incubated with either JIM4, Mac207 or LM4 as a control, washed and incubated with a secondary antibody before detecting with ECL. The tissue prints show that the different stages of *N.quercusbaccarum* express the AGPs detected by JIM4 and Mac207 as shown in Figure 5.22 (A) and (B) respectively. The distribution appears to be throughout the inner-gall and the gall cortex. There is a more intense signal

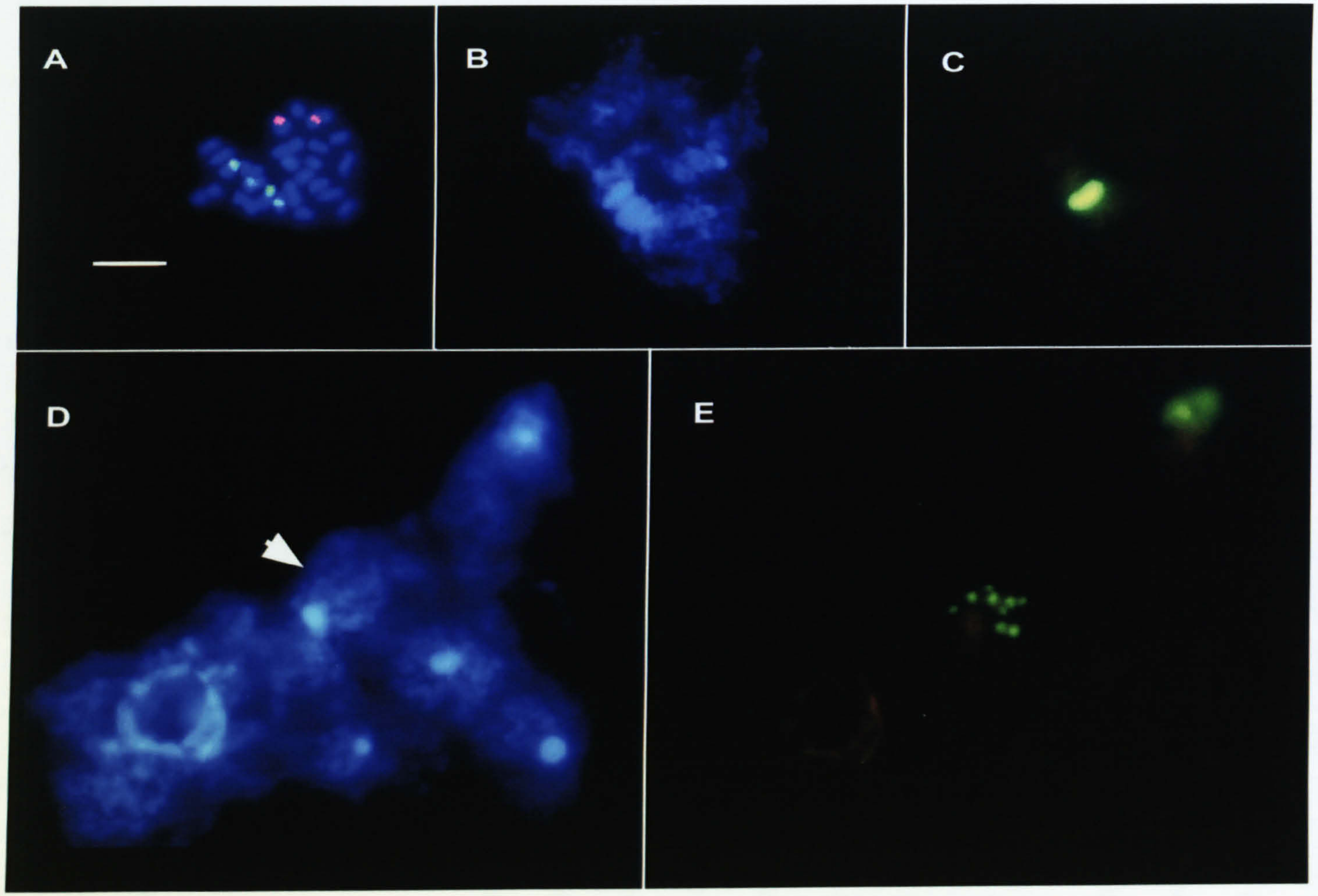


Figure 5.21 FISH of 18s, 5.8s, 26s rDNA on oak root tip nuclei and *B.pallida* inner-gall nuclei. (A) *Q.robur* root tip nuclei stained with DAPI showing 4 loci of18s, 5.8s, 26s rDNA (green) and 2 loci of 5s rDNA (red). (B) *B.pallida* polytene chromosome stained with DAPI. (C) FISH of *B.pallida* polytene chromosome showing amplified 18s, 5.8s, 26s rDNA (green). (D) *B.pallida* polytene nucleus stained with DAPI. (E) FISH of *B.pallida* polytene nucleus showing amplification of 18s, 5.8s, 26s rDNA (green).
Scale bar = 50μm

around the epidermis, however, this is also seen to some degree in the negative control LM4 (C), suggesting that it may be non-specific. The *B.pallida* galls only clearly express Mac207 at one stage (D). This is at stage 3 and the signal appears mainly in the cortical parenchyma, and the inner gall made up from a fan of chambers, shows no signal. There is, however, one clear chamber showing an intense signal, indicated by an arrow. LM4, a negative control, shows no binding (E), suggesting the signal seen in (D) from Mac207 is specific.

The antibodies were also used for immunohistochemistry as described by Smallwood *et al* (1994) on tissue sections of galls, however, this was unsuccessful. The further optimisation of the immunohistochemistry, however, will enable the differential expression of AGPs throughout gall development to be investigated. This will extend our understanding of gall development, revealing the cell markers indicative of cell type and will allow cell differentiation to be followed, perhaps even before specific cell types have fully differentiated. This would contribute also to our understanding of how the insect is redirecting plant development, from one pathway to another.

5.7 Summary of cytological changes throughout gall development

The work carried out here has further investigated the cytological changes throughout gall formation in several species of cynipid gall. The tissue section analysis has shown that, from the galls analysed here, there appears to be two main patterns of development. Both involving the enlarged lipid filled cell, although at different stages of the development. *Andricus* galls appear to feed from the cells lining the egg-like structure in the early stages, then feed from smaller cells. The *B.pallida,*

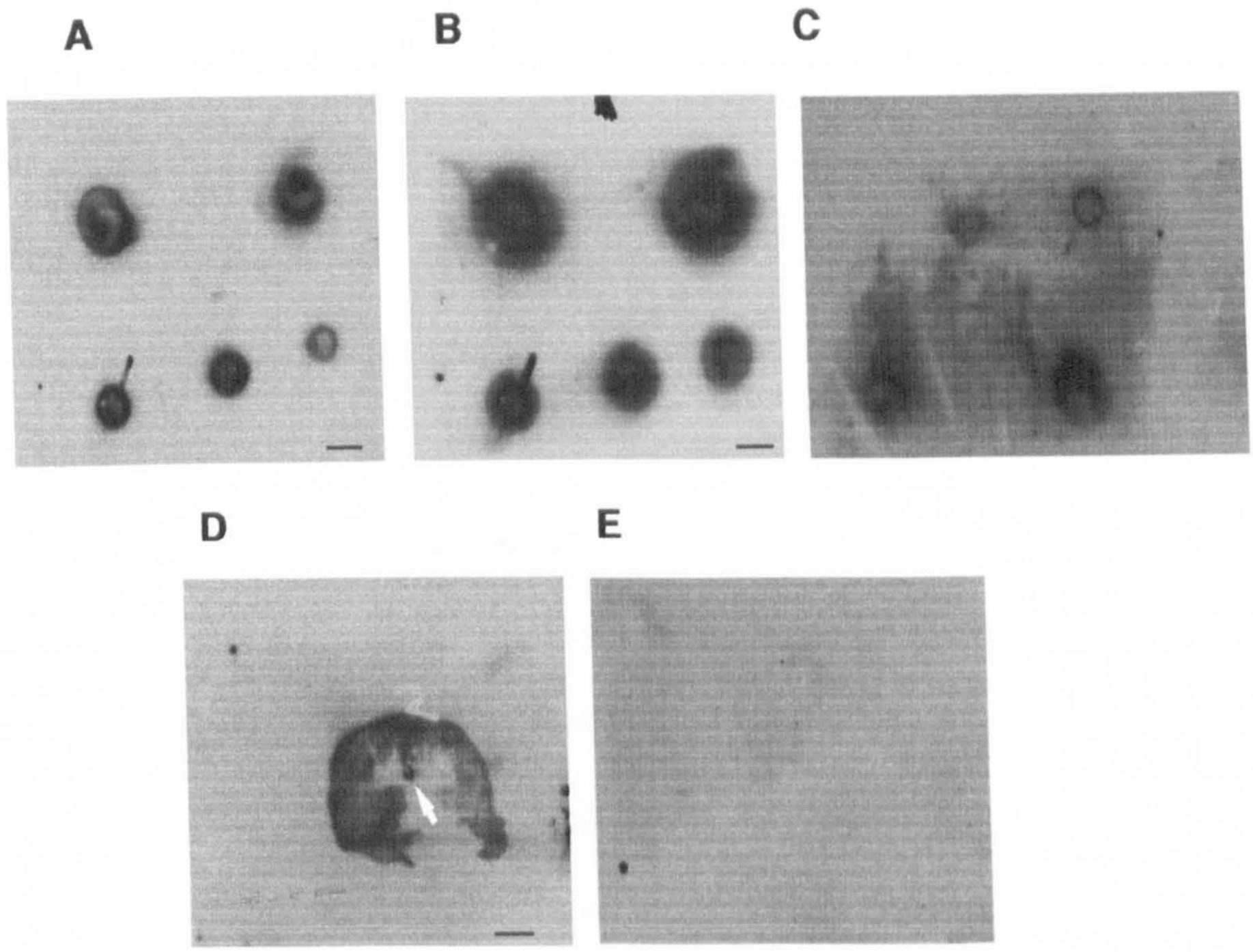


Figure 5.22 Western blots using JIM4, MAC207 and LM4 antibodies on tissue prints of *B.pallida* and *N.quercusbaccarum* galls. A) *N.quercusbaccarum* at stages 1-3 hybridised with JIM4. B) *N.quercusbaccarum* stage 1- 3 hybridised with MAC207. C) *N.quercusbaccarum* stage 1- 3 hybridised with LM4. D) *B.pallida* stage 3 hybridised with MAC207. E) *B.pallida* stage3 hybridised with LM4. Scale bar = 1cm

N.quercusbaccarum, C.quercusfolii and *D.Rosae* galls all form layers of enlarged lipid filled cells and feed from these throughout development. The induction of the nutritive cell with several distinct characteristics, in all the species tested provides an ideal marker for gall development.

The expression of FDH discussed in chapter 4, has been detected in *A.quercuscalicis* and *A.fecundator* inner-gall tissue, however, was not detected in *D.rosae*, *D.spinosa* or *A.kolleri.* Here we have shown the inner-gall tissue of *A.quercuscalicis* and *A.fecundator* differs from that observed in *D.rosae.* This could explain why the expression of FDH is detected only in these galls. The inner-gall cells are smaller than those observed in *D.rosae*, and therefore, may be under stress, coping with the increased demand of the cell. The *D.rosae* cells are enlarged and become endoreduplcated in order to cope with increased metabolic activity, and therefore are not under the same stress, so do not express FDH. *A.kolleri* was not used for tissue sections here, although it would be interesting to investigate its pattern of development to determine if it follows that observed in *A.quercuscalicis* and *A.fecundator* or *D.rosae*, as it does not appear to express FDH suggesting enlarged cells are induced.

The putative BCCP is expressed throughout all the stages of development, as detected in the western analysis of inner-gall tissue protein extracts. The spatial distribution of putative BCCP expression is concentrated in the inner-nutritive cells, indicating the lipid synthesis is taking place within the cells to provide nutrients for the larva. There appears to be greater expression in the cells immediately surrounding the larva, which decreases in the cells further from the larva, with little or no expression in the nutritive

cells on the outer part of the chamber.

Protoplast analysis and *in-situ* hybridisation, revealed the polytene nuclei in the inner-gall cells of *B.pallida*, suggesting that the cell is increasing the DNA content to cope with the increased metabolic demand.

AGP expression needs further investigation, although there does appear to be different AGP expression on the gall tissue to normal non-gall oak tissue. Analysis of additional AGP antibodies, on tissue sections throughout gall development would provide a number of cell markers which could be used to follow gall formation.

6: Development of a gall formation bioassay

6.1 Introduction: Rationale for the development of the bioassay

Concurrent with the analysis of proteins specifically expressed in inner-gall tissue, I developed a bioassay to test whether larval extracts would induce inner-gall specific proteins in a variety of plant tissues *ex-vivo* including callus, buds, leaves and stems. Bioassays have been used in previous gall formation research on non-cynipid gall formers, as discussed in Chapter 1. Previous bioassays have relied mainly on observation of accelerated growth or callus formation after application of the extract as a marker for activity, and have not used markers specific to gall formation. Assaying simply for accelerated growth or callus formation although it can identify active molecules, these may not reveal molecules specifically involved in gall formation. A molecular marker such as induction of specific genes or proteins is required as an indicator of induction of gall formation, making the bioassay both sensitive and specific.

6.2 Experimental strategy

To improve the analysis of signals involved in gall formation, we set out to create a more specific, gall formation-orientated bioassay. To achieve this I needed to consider the source of the signal to be tested for activity, the target tissue used to assay activity and the markers used to indicate activity. The molecular markers are essential for the assay as these are indicative of gall formation activity or a more general response. As the markers, I chose inner-gall proteins and their induction in the target tissue of the assay. Analysis of inner-gall tissue from different species of cynipid galls throughout their development was being conducted concurrent with the bioassay development and therefore it seemed appropriate to use protein induction as the assay. The source of the cynipid gall-forming

signal is the larva and not the ovipositional fluid secreted by the adult female, as is true for some non-cynipid galls discussed in Chapter 1. The larva was therefore used as the source of the active morphogen, the preparation of which will be discussed in the next section. The tissue exposed to the larval extract in the bioassay must be responsive to active signals. After exposure to the larval extract, induction of additional proteins within the target tissue can be analysed by carrying out a protein extract and running on a gradient SDS-PAGE gel. Using proteins as markers, I set out to isolate the active compounds of the extract, with the eventual objective of identifying the gall forming signals.

Results

6.3 Preparation of cynipid larval extract as the source material for larval signals.

Overwhelming evidence suggests the larva is secreting the signal for the induction and maintenance of gall formation. Upon death of the larva, the enzyme activity within gall tissue declines and the gall ceases development. To isolate the active morphogen secreted by the larva, I decided to use larval extract and test for activity. Total larval extract was chosen over collecting actual secretions of the larva as this is a difficult process and the volume of secretion is too small for the style of bioassay used here. To make the extract, the larvae were carefully dissected from the developing galls and ground using dry ice and mixed with water to form the extract (see section 2.5.3).

Effort was taken to prepare fresh extract whereever possible although extracts prepared and stored at –20°C appeared to retain activity.

6.4 Evaluation of target plant tissue for use in bioassay

The type of tissue used in the bioassay is fundamental to the sensitivity of the assay. The tissue must be responsive to external signals and free of other contaminants such as fungal or bacterial infection. For a gall formation bioassay an actively proliferating tissue such as callus is ideal as it resembles the meristematic tissue on which the eggs are laid by the adult wasp, it is responsive to external signals, sterile and easily propagated. Oak callus tissue was formed from *Q.robur* stem tissue (see section 2.5.1). Great difficulty was experienced producing the callus, due to the high level of fungal spores within the oak vascular system. Surface sterilisation was not sufficient to remove contaminants and extensive sterilisation protocols were carried out before oak callus was obtained. Once obtained, attempts were made to set up liquid cultures, however, these were not successful. The oak callus preferred solid media and was slow growing. When exposed to the larval extract this did not give induction of protein, perhaps owing to the low cellular activity. This was therefore not used in the bioassays.

Rose callus was formed from leaves of *Rosa rugosa*. Surface sterilisation of the leaves was carried out before leaf discs were cut from the leaves and placed on nutrient MS media. Callus formation from the wounded edge of the leaf took several weeks and once established this was carefully removed from the leaf tissue and cultured in liquid media on a rotating platform at 24°C. The callus was regularly subcultured until required for the bioassay. The rose callus was responsive to the larval extract possibly because it was faster growing than the oak callus. When exposed to the larval extract and the protein extract run on an SDS-PAGE gel, an induced band can be seen at 62kDa as shown in

Figure 6.1.

To determine if host plant organs, on which galls can be formed, are responsive to the extract, *Q.robur* buds, leaf discs and midribs were used in the bioassay and exposed to *B.pallida* larval extract. Protein extracts of the exposed tissues and non-exposed controls were analysed using SDS-PAGE. Induced proteins at 87.25kDa and 62kDa can be seen in the bud, midrib and leaf, as shown in Figure 6.2. The 62kDa band is fainter in bud and the 87.25kDa band fainter in midrib and leaf disc compared to bud. The induction of the same 62kDa band in plant organs as seen in the callus tissue demonstrates that the extract is active and after exposure with several types of plant tissues, can induce the same protein.

To choose the optimal target tissue for the bioassay additional factors such as availability need to be considered as well as the responsiveness of the tissue. All tissues are responsive, however bud, leaf and midrib tissues are only available in spring and summer, and one would expect that as the leaves mature the responsiveness of the tissue would decline. The buds and leaves would also need to be collected immediately prior to use in the bioassay, which is inconvenient and the possibility of herbivore damage or fungal contamination is high. Rose callus is both readily available in high quantities throughout the year and free from contamination, and for these reasons was chosen as the target tissue for the bioassay.

6.5 Comparison of proteins induced in rose callus tissue by different cynipid species

Initial bioassay experiments were carried out using *D.spinosa* larva, sent from Canada by Joe Shorthouse. *D.spinosa* is not readily available at the field sites used for gall collection in England, therefore, alternative, more abundant galls were chosen. *B.pallida* and *A.quercuscalicis* are both oak galls abundant at the field sites in spring and summer respectively and larval extracts from these were used in the remaining bioassays. Restrictions of gall forming seasons and limited storage space, precluded our using the same species throughout all the bioassays. The availability of rose galls also constrained me from using rose galls and meant larvae from oak galls would be used on rose callus in the bioassay.

Bioassays with the larvae from oak galls showed that both larval extracts did induce proteins after exposure to rose callus, however, these varied depending on species as can be seen in Figure 6.6. *D.spinosa* induced a 72kDa and 57ka bands, *A.quercuscalicis* induced 66kDa band and *B.pallida* induced 87.25kDa and 62kDa bands. The difference between the larval species is not surprising as they all induce very different galls, and the protein signatures of the inner-gall tissue all vary as discussed in Chapter 4. It is unlikely that the difference is due to the test tissue being rose and the host tissue of *B.pallida* and *A.quercuscalicis* is normally oak. It would be preferable if there was a common band induced by all the species, as this would indicate a common signal used by all which is what we are attempting to elucidate. Despite the variability between cynipid gall species, a common signalling pathway is probably used during the gall formation process, and slight modifications to this by each species enables species-specific galls to be formed. Hopefully, the development of this specific bioassay will eventually help in the

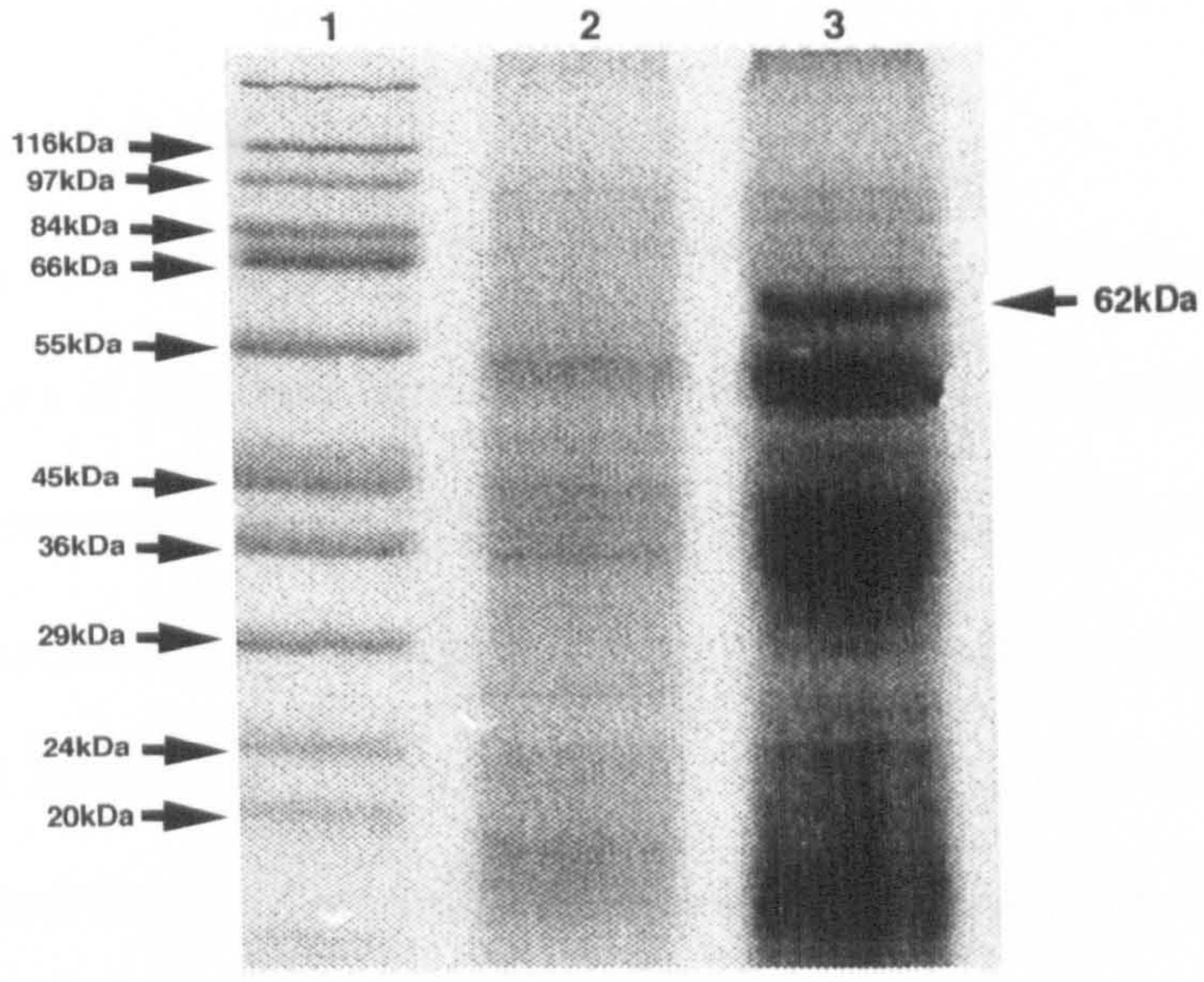


Figure 6.1 A gradient SDS PAGE gel (6.5%-20%) showing callus protein extract after using B.pallida larval extract. Lane 1 Wide range molecular weight marker. Lane 2 Callus protein extract, not exposed to extract. Lane 3 Bioassay using B.pallida larval extract with induced 62kDa band.

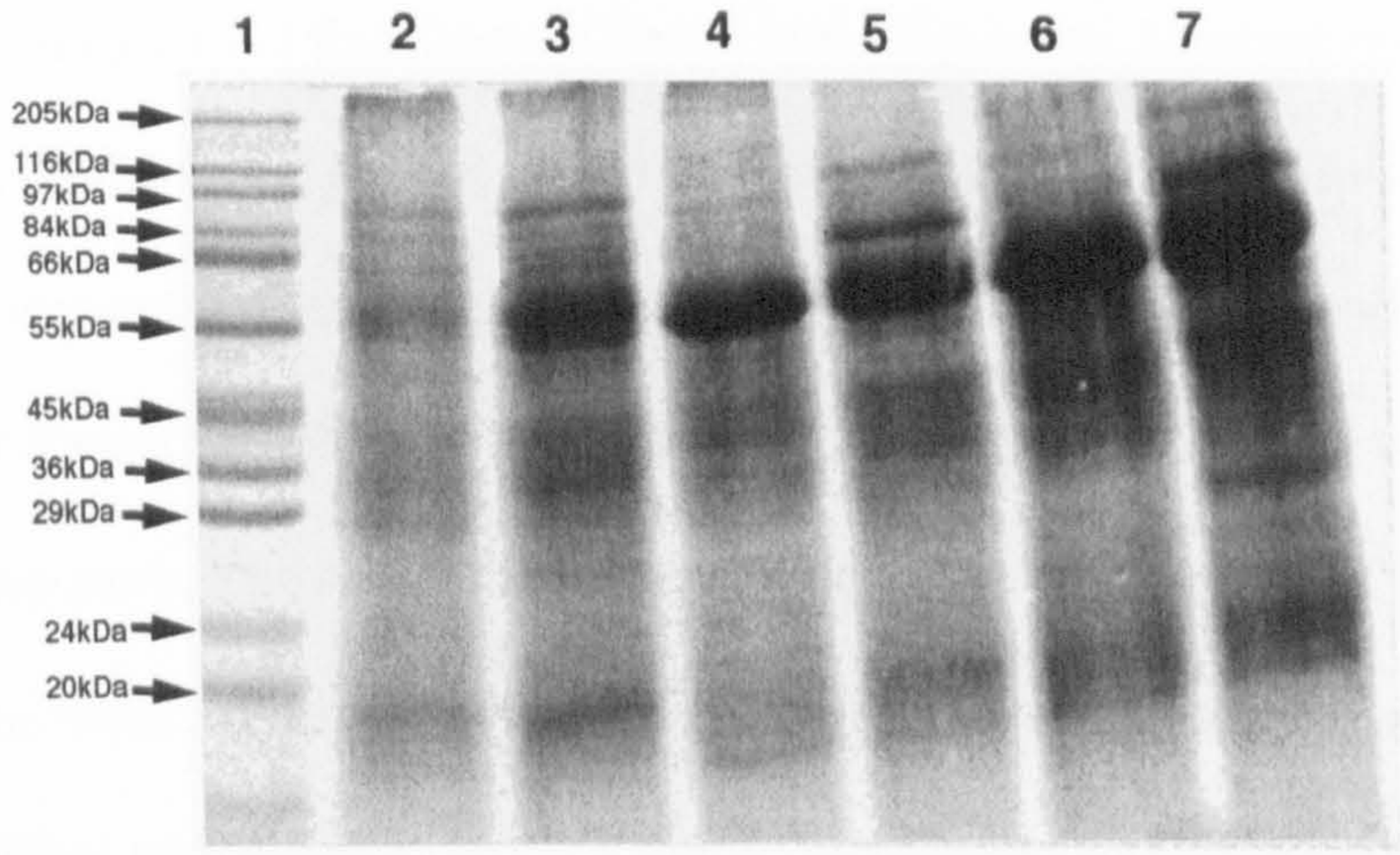


Figure 6.2 A gradient SDS PAGE gel (6.5%-20%) showing oak bud, leaf and midrib protein extracts after a bioassay using *B.pallida* larval extract. Lane 1 Wide range molecular weight marker. Lane 2 Bud protein extract. Lane 3 Bud protein extract after bioassay. Lane 4 Midrib protein extract. Lane 5 Midrib protein extract after bioassay. Lane 6 Leaf protein extract. Lane 7 Leaf protein extract after bioassay.

elucidation.

6.5.1 Optimal length of exposure to larval extracts

The length of time the callus tissue is exposed is critical as sufficient time is required to allow induction of proteins to occur and for them to reach detectable levels. Equally, too long an exposure may lead to decreasing levels of protein, which can no longer be detected. Experimentally, the shortest possible exposure time, which still allows induction of proteins is favourable. To determine the length necessary, several time exposures were carried out, and the shortest which showed induction was chosen. This was an over night exposure of approximately 16 hours (results not shown).

6.5.2 Titration of larval extract

Later experiments would involve the fractionation of the extract, therefore, it was important to test at what concentration the extract was active to ensure a high enough concentration of the fractions was used in the bioassay. Limited supply of gall material also meant the larval extract had to be used efficiently. To test at what concentrations the extract was active, several concentration of *A.quercuscalicis* larval extract were tried, to find the lowest possible concentration that initiated the induction of protein in an over night exposure. The standard extraction procedure uses 300mg of larva in 500µl of water. The bioassay was set up using the standard procedure (section 2.5.4) and 50µl, 100µl and 200µl of larval extract. Protein extracts of the exposed callus and control callus were carried out and run on a SDS-PAGE gel. Figure 6.3 shows the protein extract of callus after a bioassay. Lane 4 shows the bioassay using only 50µl of the extract and an induction of a 66kDa band can be seen, although less intense than in

callus only, not exposed to larval extract. Lane 4 Bioassay using 50µl *A.quercuscalicis* filtered extract. Lane 5 Bioassay using 100µl *A.quercuscalicis* filtered extract.Lane 6 Bioassay using 100µl *A.quercuscalicis* unfiltered extract. Lane 7 Bioassay using 200µl *A.quercuscalicis* filtered extract.lanes 5, 6, and 7. The total protein content, however, appears slightly lower in lane 4 which may account for the lower induction. Lane 5, 6 and 7 all appear to have an induction of similar intensities. Despite the lower level of induction we decided to use 50µl of the standard extract, as it does give an induction of a 66kDa band and the small volume means the limited material will be used efficiently.

6.6 Is the induced protein related to gall formation ?

6.6.1 Do leaf miners induce the same proteins?

The previous experiments have shown that there is an induction of protein in response to the extract, to ensure that the induction is specific to the extract and not a general defence response to herbivores, bioassays using non-galling herbivores, such as the leaf miner *Profonuca pygmea,* were carried out. The *P.pygmea* larval extract was applied to callus tissue in parallel to the cynipid larval extract for 16 hours and before protein extraction of callus. Protein analysis on SDS-PAGE gels in Figure 6.4.A. shows the leaf miners did cause induction of a protein; however, this was different from the protein induction seen with cynipid wasps. The induction of an intense 84kDa protein by *P.pygmea.* can be seen in lane 4, which differs from the induction of a 62kDa band caused by *B.pallida* extract as seen in lane 3. The difference in the size of induced bands suggests that the plant is not showing a general defence response to the cynipid larval extract. A plant's primary defence response is general and we would not expect to see specific proteins to different herbivores so soon after exposure.

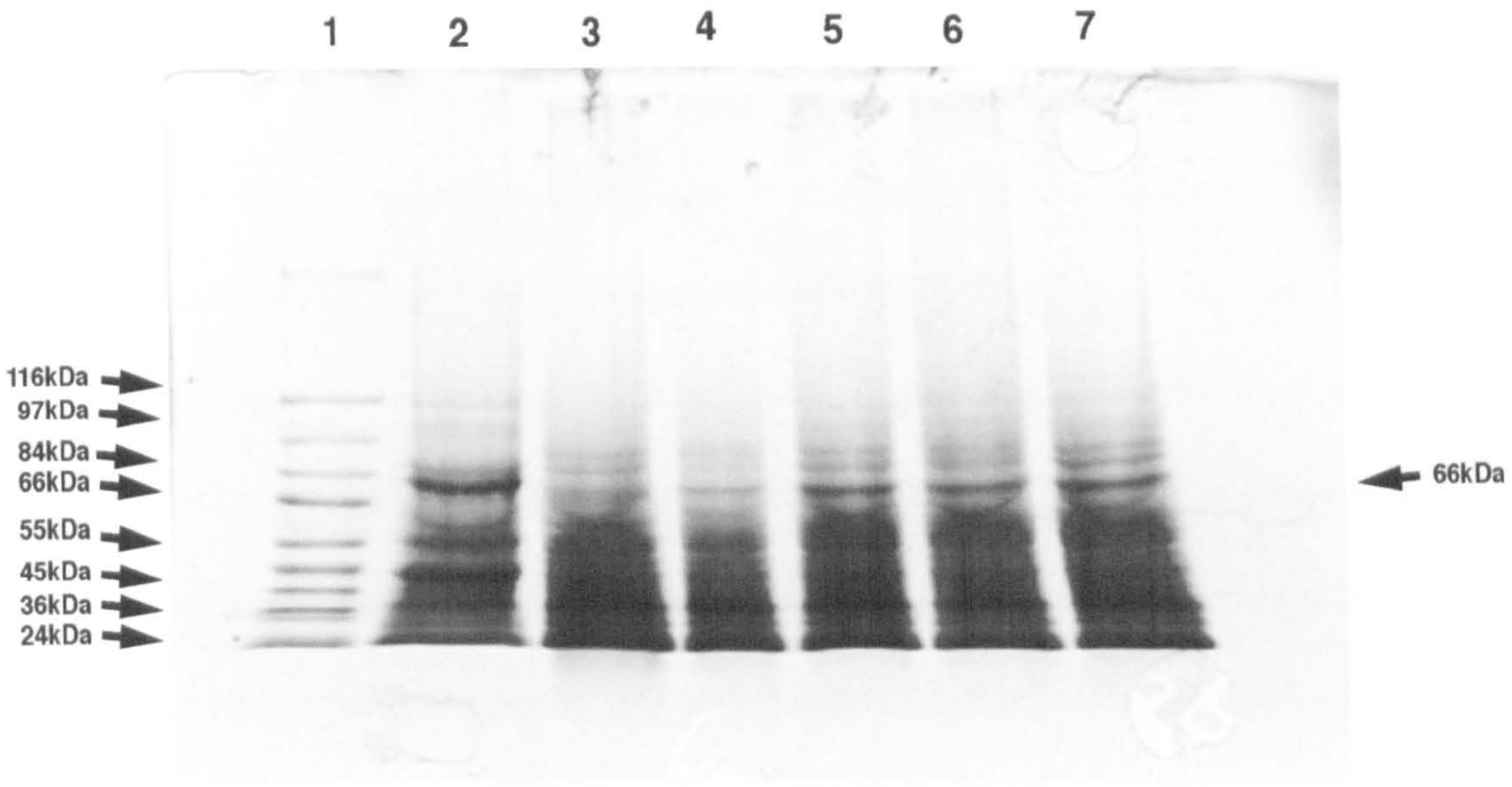


Figure 6.3 A gradient SDS PAGE gel (6.5%-20%) showing callus protein extracts after bioassay using different concentrations of *A.quercuscalicis* larval extract.
Lane 1 Wide range molecular weight marker. Lane 2 *A.quercuscalicis* inner-gall tissue. Lane 3 callus only, not exposed to larval extract. Lane 4 Bioassay using 50µl *A.quercuscalicis* filtered extract. Lane 5 Bioassay using 100µl *A.quercuscalicis* filtered extract.Lane 6 Bioassay using 100µl *A.quercuscalicis* unfiltered extract. Lane 7 Bioassay using 200µl *A.quercuscalicis* filtered extract.

6.6.2 Verification that induced proteins are of plant not cynipid origin

It is possible that we are seeing proteins from the actual larval extract applied to the callus tissue and not the induction of a protein. In an attempt to rule this out we analysed the *B.pallida* and *P.pygmea* larval extracts on SDS-PAGE to determine if bands the same size as induced in the bioassay were present. Figure 6.4.B. shows the *B.pallida* and *P.pygmea* larval extract and both show several intense bands. In the *P.pygmea* extract, although overloaded, displays a very prominent band at 80kDa and another a 24kDa. The band induced in the bioassay by this larval extract is 84kDa, which is close to the intense band in the extract. It is difficult to determine if the 84kDa band in the bioassay is from induction, however, the sizes are slightly different and other intense bands in the extract are not seen in the bioassay. The *B.pallida* larval extract has a very intense band at 52-56kDa, 31kD and 18.8kDa. Many less intense bands can be seen including 89kDa, 79.5kDa, 66kDa and 64kDa and 48.3kDa. The induced band is 62kDa, which is not seen in the larval extract, however, bands at 64kDa and 66kDa are present. Again it is difficult to determine if the induced band is not the larval extract but other intense bands are not seen in the bioassay. The 87.25kDa band, sometimes seen in the *B.pallida* bioassay, is not seen here and is not always induced in the bioassay. In the extract, however, there is not a band present at 87.25kDa. The fact that the 87.25kDa band is not always observed suggests that the band(s) observed are of true induced protein and not that of the larval extract. If it were the larval extract we would see 87.25kDa in each bioassay. To investigate further the true induction proteins other methods were carried out.

6.6.3 Biotinylation of larval extract

To confirm that the extra bands are induced in response to the extract, a bioassay using

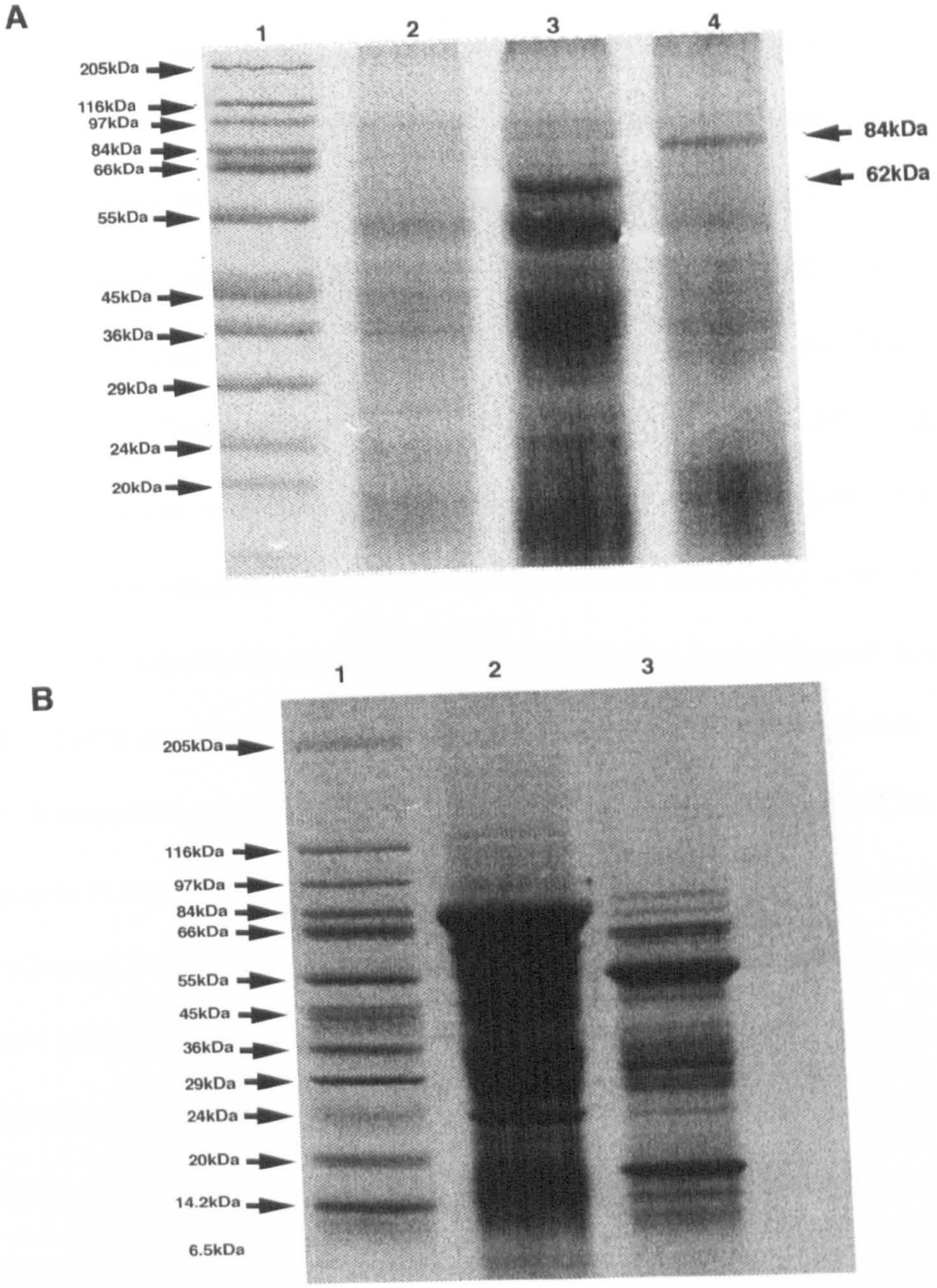


Figure 6.4 SDS PAGE gradient gels (6.5%-20%) showing (A) callus protein extracts after a bioassay using *B.pallida* larval extract and *P.pygmea* larval extract. (B) *B.pallida* larval extract and *P.pygmea* larval extract. In (A) Lane 1 Wide range molecular weight marker. Lane 2 Callus only, not exposed to extract. Lane 3 Bioassay using *B.pallida* larval extract showing induction of 62kDa band. Lane 4 Bioassay using *P.pygmea* larval extract showing induction of 84kDa band. In (B) Lane 1 Wide range molecular weight marker. Lane 2 *P.pygmea* larval extract. Lane 3 *B.pallida* larval extract.

we are visualising larval proteins. Biotinylation was carried out as described in section 2.5.9.1, and a standard bioassay was carried out using this extract and non-biotinylated extract. The protein extract of the exposed callus was analysed on SDS-PAGE gel with non-exposed callus and the biotinylated extract in duplicate, to enable one half of the gel to be stained with Coomassie Blue and the other blotted. One half was blotted onto Hybond C and incubated with streptavidin horseradish-peroxidase and detected using ECL. The film was compared to the Coomassie stained proteins shown in Figure 6.5.A and B. The biotinylated extract stained with Coomassie shows only a few distinct bands, however, on the film as the streptavidin detection method is significantly more sensitive and many bands can be seen in the biotinylated extract, lane 3 Figure 6.5.B. The bioassay with the biotinyated extract does show one clear biotinylated band at 76kDa. This band corresponds to one seen in the extract indicating that we may be seeing the larval extract in the bioassay protein extract. The streptavidin peroxidase detection is more sensitive than the Coomassie staining, however, the biotinylated band seen in the bioassay can also be seen when the extract is stained with Coomassie. The bioassay does not always give induction of the protein suggesting that if it is the larval extract we were observing in the bioassay, we would always see additional bands. Additionally the concentration of the larval extract is diluted 10 fold in MS media when exposed to the callus, and this is drained before carrying out protein extraction of the callus, further reducing the possibility of contaminating larval extract. The induced band in a successful bioassay is seen slightly lower than the biotinylated band at 66kDa. There is little difference in the size, however, as biotin does not affect the migration of the proteins and as none of the other prominent biotinylated bands can be seen in the bioassay, the induced protein is likely to be a separate protein.

Unfortunately, a biotinylated protein at 35kDa is not detected in the bioassay, showing that the putative BCCP protein detected in inner-gall tissue is not being induced in the bioassay. This would have provided an ideal marker for gall formation, however, with further development of the bioassay this may be possible.

6.6.4 Is the induction caused by wound response?

Pathogen attack initiates an induced defence response in plants and the wounding caused during gall formation probably initiates such a defence response. Gene expression and signalling molecules involved in such responses are well documented (Kúc 1997). Part of the plant defence response to pathogens is the expression of pathogen related (PR) proteins. These proteins are traditionally divided into 5 groups PR 1-5, with each group made up of a family of proteins, although many more have been identified to date (Shirsat *et al.*, 1996). PR-1 (15-17kDa) are thought to have antifungal activity; PR-2 are a group of ß-1,3-glucanases used to break down fungal cell walls, releasing oligosacharides which act as elicitors for further plant defense responses (Ryan and Farmer, 1991); PR-3 (33-41kDa) are a group of chitinases which are divided into six groups according to their structure (Neuhaus, 1999); PR-4 (13-14.5kDa) has four members, however, their exact function is unknown; PR-5 group of proteins are osmotin/proteinase inhibitors and are generally found extracelluarly or in the vacuole (Stintzi *et al.*, 1993). From these, the PR-3 group of chitinases are likely to be involved in the cynipid-plant interaction, due to the high chitin content of the cynipid wasp. The PR-3 group of chitinases hydrolyse the ß-1,4-N-acetylglucosamine linkages of chitin, relaeasing oligomers. These oligomers could then elicit additional defense responses. In the bioassay the induced bands do not correspond to the known sizes of PR protein, although it is likely that some PR proteins

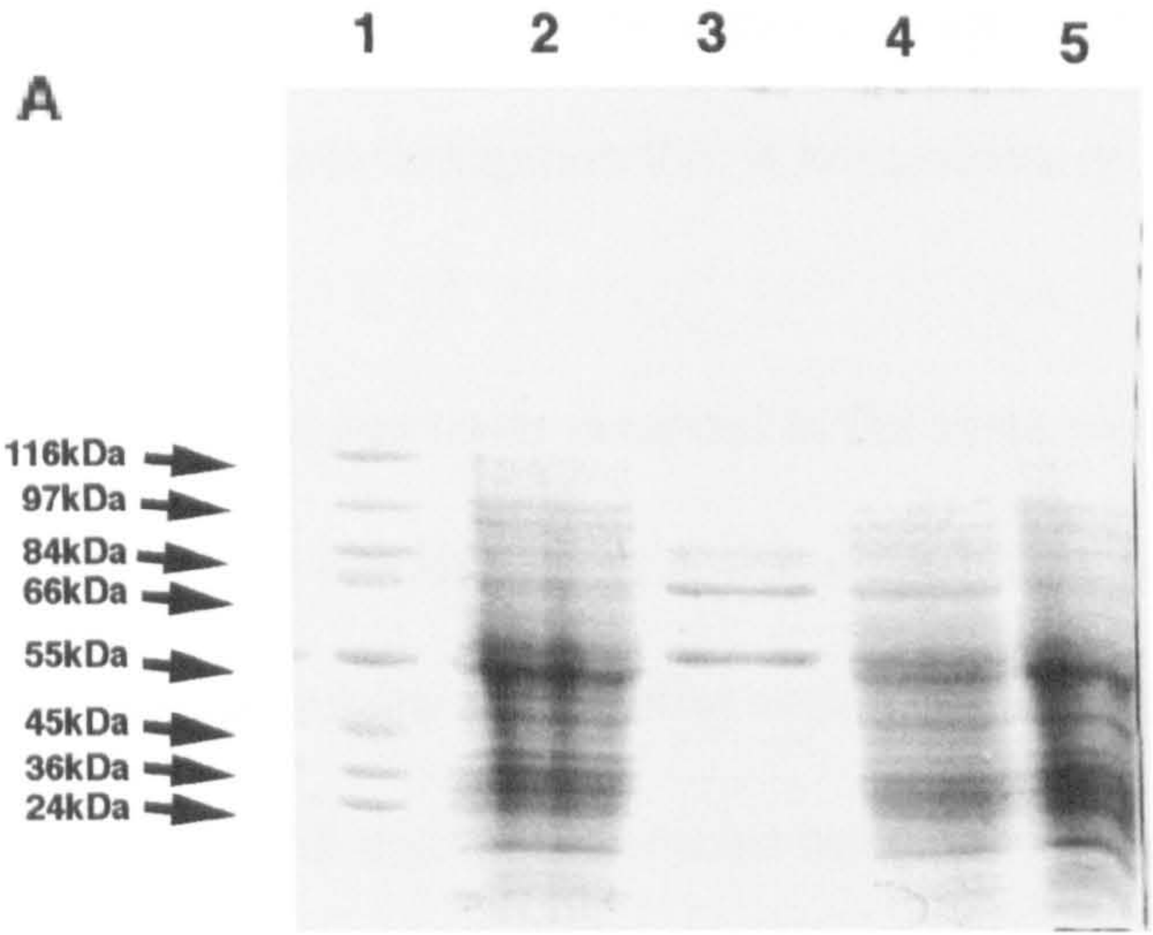


Figure 6.5.A. A gradient SDS PAGE gel (6.5%-20%) showing callus protein extracts after bioassay using *A.quercuscalicis* biotinylated and non-biotinylated larval extract. Lane 1 Wide range molecular weight marker. Lane 2 Bioassay using biotinylated *A.quercuscalicis* larval extract. Lane 3 Biotinylated *A.quercuscalicis* larval extract. Lane 4 Bioassay using non-biotinylated larval extract. Lane 5 Callus only, not exposed to extract.

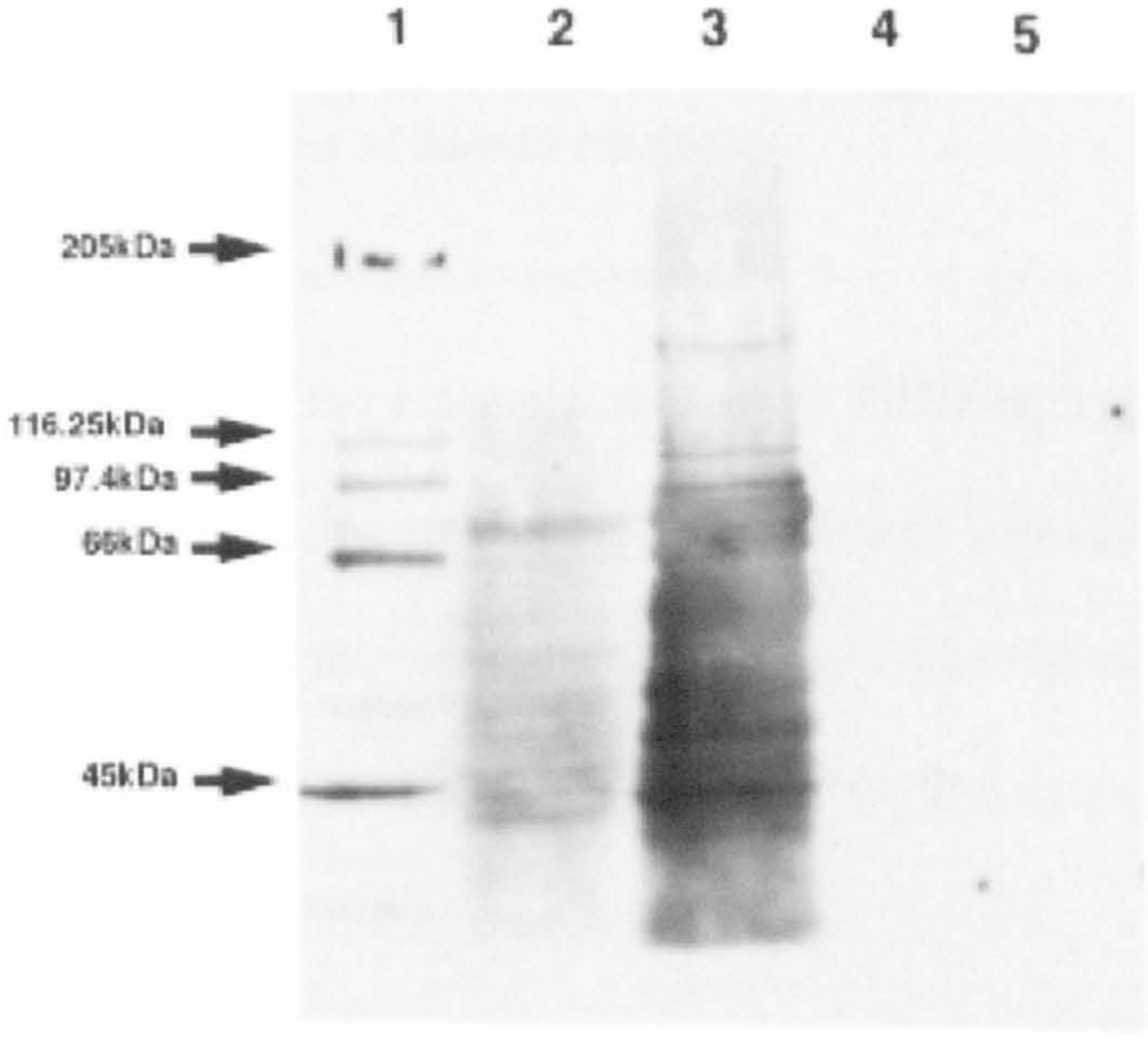


Figure 6.5.B. A Western blot of callus protein extract after bioassay using biotinylated and non-biotinylated *A.quercuscalicis* larval extract. Lane 1 Biotinylated molecular weight marker. Lane 2 Bioassay using biotinylated larval extract. Lane 3 Biotinylated larval extract. Lane 4 Callus only, not exposed to extract.

are induced. To visualise these, antibodies against the specific PR proteins could be used, however, for this investigation this is not necessary.

One molecule known to be involved in the systemic plant defence response is jasmonic acid (JA), which is a signalling molecule inducing the expression of genes involved in resistance to pathogen attack (Niki *et al*, 1998). The 3 major proteins induced by JA are JIP 23, 37, and 66 and these proteins have been induced in leaf discs by exogenously supplied JA at 4nM (Niki *et al*, 1998). To determine if this response could be achieved in our bioassay and if any of the proteins we have observed are involved in induced defence, I used JA in the bioassay at a number of concentrations between 0.1nM-2μM for 24 h and 48 h (see section 2.5.9.3). No induction was observed, suggesting that we are not seeing a defence response.

6.7 Fractionation of larval extract

6.7.1 Size fractionation of larval extract

Fractionation of the extract would allow different fractions to be tested for activity, eventually leaving a small enough fraction to identify the signal. One method used to separate the extract was size fractionation. The larval extract was first filtered through a 0.2μm syringe filter and tested for activity. The extract retained its activity indicating that the molecule is smaller than 0.2μm.

Further size fractionation was carried out using microcon spin columns with 30kDa and 100kDa cut off points to fraction the extract according to size (see section 2.5.5). Once separated according to size the fractions were testing using the bioassay. The active

fractions were those >30kDa and <100kda. Figure 6.6.A shows the induction of proteins with molecular weights of 72kDa, 57.2kDa and 55kDa, by fractions of *D.spinosa* extract >30KDa and < 100KDa on rose callus. This indicates that the active molecule(s) is >30KDa and <100KD. The control with no extract, however, also has a 57.2kDa protein which suggests that only the 72kDa and 55kDa protein are actually induced by the extract. Interestingly the callus only sample is expressing the 57kDa band at higher levels than the lane 2 and 5.

The *B.pallida* larval extract size fractionation also show that the >30KDa and <100KDa fraction gives induction as shown in Fig. 6.6.B. Although not as clear as the results obtained from *D.spinosa*, it is possible to see that the >30KDa fraction gave an induction of 87kDa, 62kDa and 57KDa sized bands. The callus only does not show the expression of 87kDa protein. These proteins can be compared to the inner-gall tissue of *B. pallida* in lane 2. This shows an abundant proteins at 91kDa and 55kDa and a very faint one at 58kDa. This does not mean that proteins induced are the same as the inner-gall protein, only of similar molecular mass.

A. quercuscalicis larval extract >30kDa gives the induction of a 66kDa protein as shown in Figure 6.6.C(i). This can be compared to the *A. quercuscalicis* inner-gall tissue in Figure 6.6.C(ii). The inner-gall tissue shows distinct bands at 70kDa, 56kDa and 44kDa. The larval extract in lane 3 shows clear bands at 90kDa, 62kDa, and 56kDa.

6.7.2 Is the active molecule in the head or body of the larva

In an attempt to identify where in the larvae the morphogen is produced I separated the

head of the larva, containing the salivary glands, and the body of the larva, containing the malpighian tubule. The malpighian tubules are similar to kidneys and it has been suggested that these produce and secrete the morphogen. The salivary glands are an obvious place for the production and secretion of the morphogen as larvae feed on the inner-gall tissue transforming nutritive parenchyma into nutritive tissue. An extract was obtained from the different sections of *A.quercuscalicis* larva and used for a bioassay. A pooled fraction of the head and body larval extracts was also used to ensure the extract was still active if no induction was seen from either fraction separately. Induction of the 66kDa band was obtained from the pooled fraction and the body fraction, suggesting the body contains the active molecule as shown in Figure 6.7.

6.7.3 Is the active molecule a sugar or a glycoprotein?

To determine if the active molecule was a sugar or a glycoprotein, I used a Concanavalin A (ConA) column that allows the separation of sugars by binding molecules that contain α-D-mannose and α-D-glucose. The crude larval extract was put through the ConA column and the bound polysaccharides, glycoproteins, or glycolipids were eluted using different molarity of eluent. As the molarity increases so does the affinity to which the sugar binds to the column. The flow through, of nonbound molecules, and the different

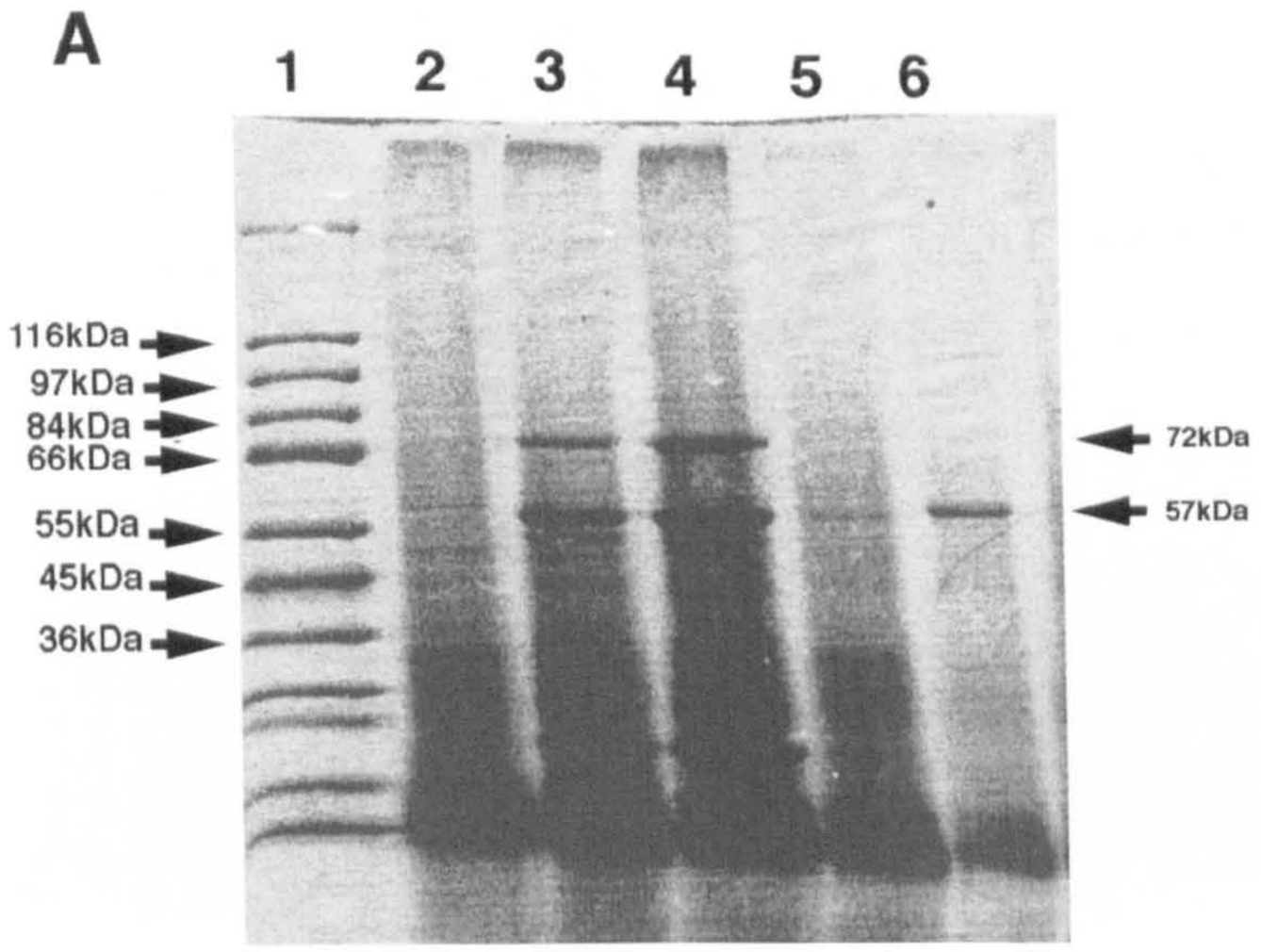


Figure 6.6.A. A gradient SDS PAGE gel (6.5%-20%) showing callus protein extracts after bioassay using size fractionated *D.spinosa* larval extract. Lane 1 Wide range molecular weight marker. Lane 2 Bioassay using *D.spinosa* larval extract <30kDa. Lane 3 Bioassay using *D.spinosa* larval extract >30kDa. Lane 4 Bioassay using *D.spinosa* larval extract <100kDa. Lane 5 Bioassay using *D.spinosa* larval extract >100kDa. Lane 6 Callus only, not exposed to extract.

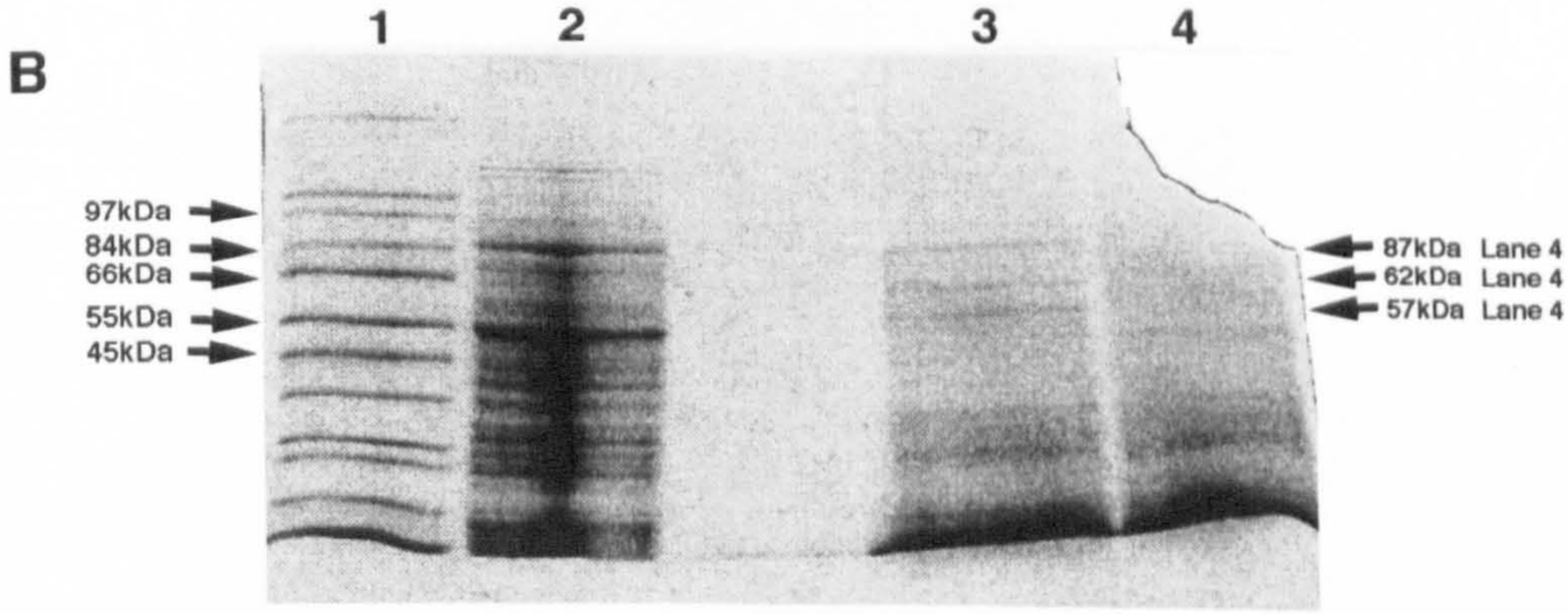


Figure 6.6.B. A gradient SDSPAGE gel (6.5%-20%) showing callus protein extracts after bioassay using size fractionated *B.pallida* larval extract. Lane 1 Wide range molecular weight marker. Lane 2 *B.pallida* inner-gall protein extract. Lane 3 Bioassay using *B.pallida* larval extract >30kDa. Lane 4 Bioassay using *B.pallida* larval extract <30kDa

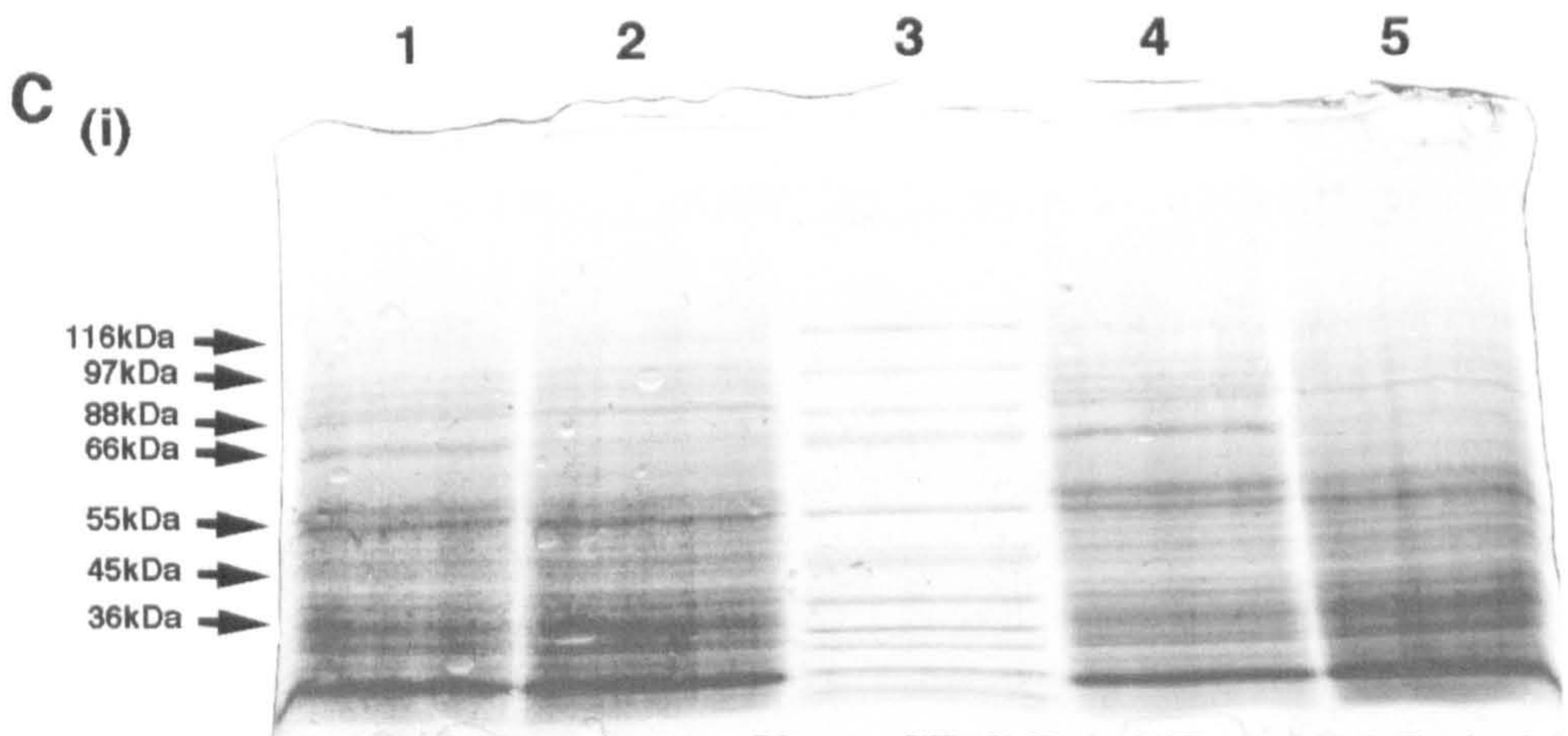


Figure 6.6.C(i). SDS-PAGE gradient gel (6.5%-20%) showing callus protein extracts after bioassay using size fractionated *A.quercuscalicis* larval extract. Lane 1 Bioassay using *A.quercuscalicis* larval extract using <30kDa. Lane 2 Bioassay using *A.quercuscalicis* larval extract >30kDa.Lane 3 Wide range molecular weight marker. Lane 4 Bioassay using total *A.quercuscalicis* larval extract. Lane 5 Callus only, not exposed to extract.

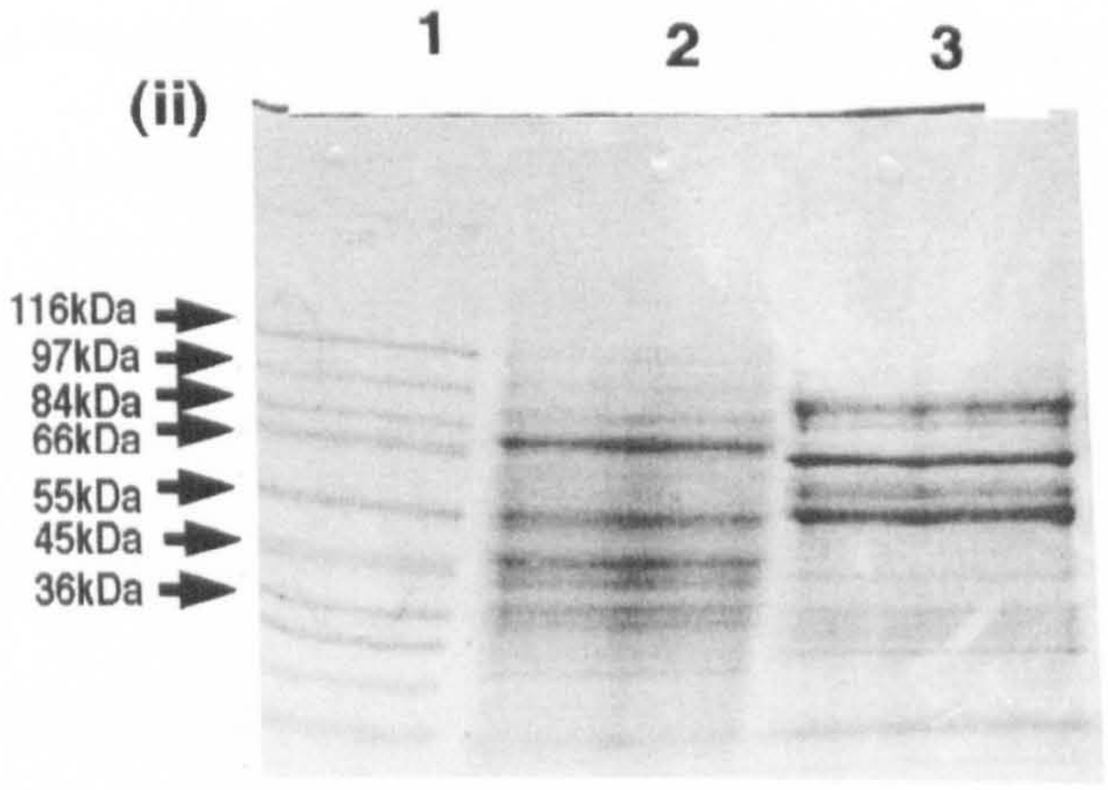


Figure 6.6.C(ii) SDS-PAGE gradient gel (6.5%-20%) showing *A.quercuscalicis* inner-gall tissue and larval extract. Lane 1 Wide range molecular weight marker. Lane 2 *A.quercuscalicis* inner-gall protein extract. Lane 3 *A.quercuscalicis* larval extract.

fractions collected were tested in the bioassay; however, none produced a positive induction. This result could be explained by the active morphogen binding so strongly to the column that even at a high molarity the buffer could not elute the molecule from the column or the active molecule may have been deactivated during the separation process.

6.7.4 Is the active molecule a protein?

The larval extract itself has a high protein content and as the ConA column results did not indicate whether the active molecule is a protein or sugar, to determine if one of the morphogens is a protein, anion exchange chromatography was used to separate the proteins. The negatively charged molecules from the larval extract bind to the positively charged column with varying affinities and they are then eluted from the column using a series of salt buffers with a gradual increase in salt concentration. The affinity of the protein to the column is proportional to the concentration of salt in the elution buffer. The extract was put through a Mono Q anion exchange column and fractions were collected according to peaks of absorbency at 280nm. Figure 6.8 shows two traces (A) and (B) of the extract when put through the column, the first time (A) using a gradient of salt elution buffer up to 500mM and the second time (B) using a steeper gradient of salt elution buffer up to 1M NaCl. The samples fractionated in (A) were concentrated and salt removed using a spin column to ensure that the salt did not interfere with the bioassay and the fractions were concentrated to the correct concentration used in the original bioassay. These fractions did not appear to induce any proteins as seen in the control of the crude extract. This would indicate that the molecule is either a non-protein substance, positively charged protein at pH7, or the whole procedure may have inactivated the molecule. It is possible that the protein

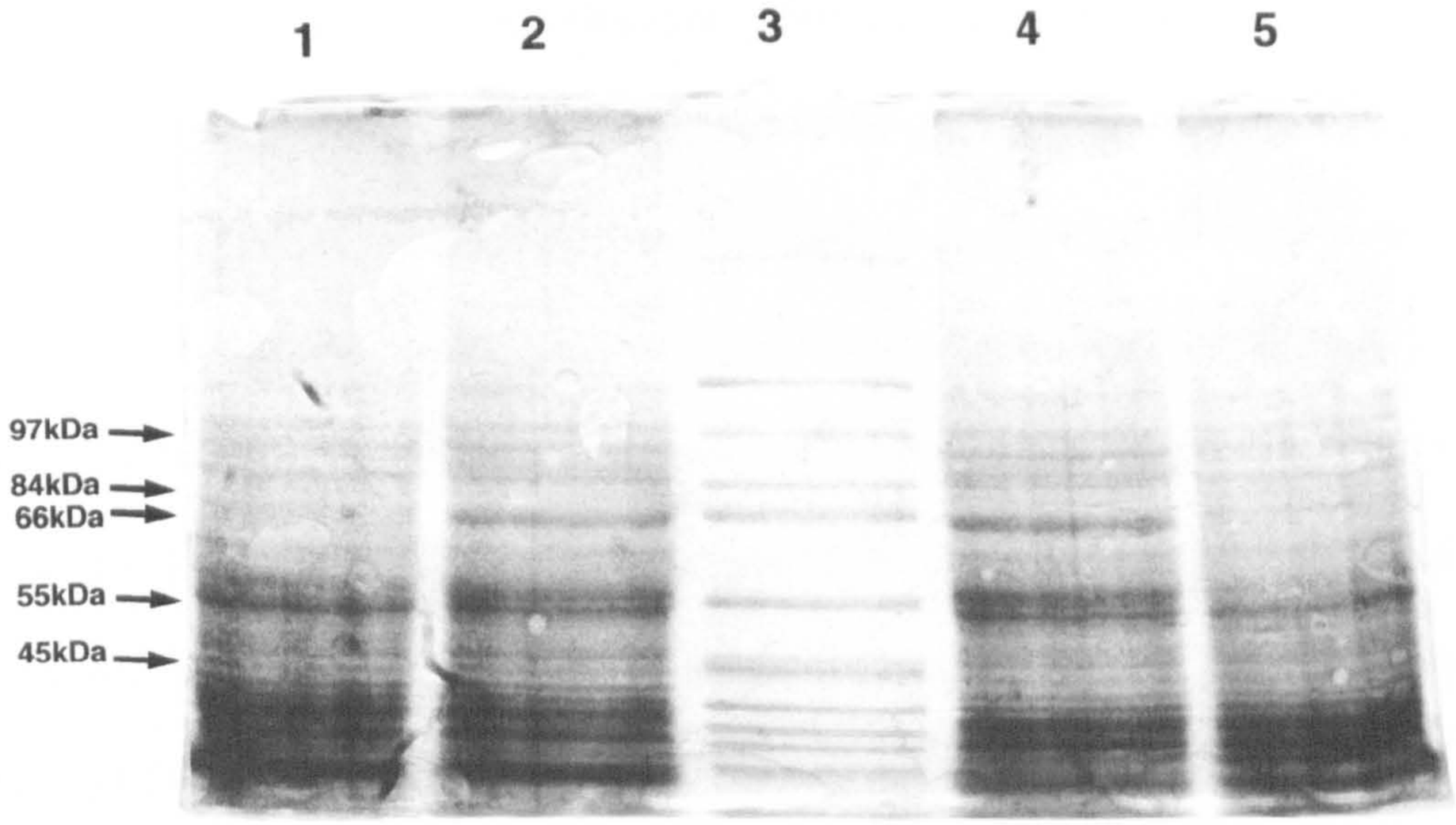


Figure 6.7 A SDS-PAGE gradient gel (6.5%-20%) showing callus protein extracts after bioassay using *A.quercuscalicis* head and body larval extract. Lane 1 Bioassay using *A.quercuscalicis* larval head extract. Lane 2 Bioassay using *A.quercuscalicis* larval body extract. Lane 3 Wide range molecular weight marker. Lane 4 Bioassay using pooled *A.quercuscalicis* head and body larval extract. Lane 5 Callus only, not exposed to extract.

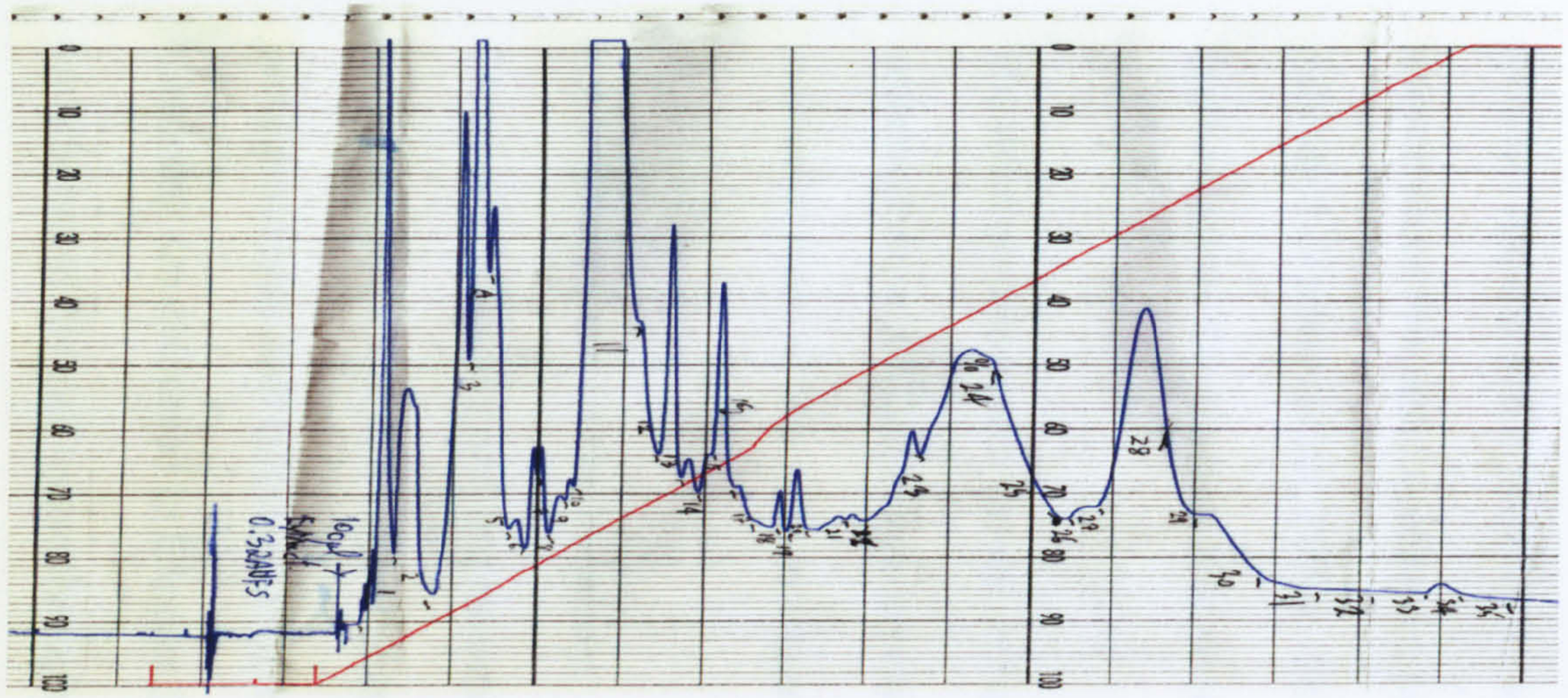
needs a co factor to be active, and therefore by fractionating the activity is lost. The active molecule may have been in the immediate flow through, as the fractions were not collected until the first peak appeared on the trace.

The second ion exchange fractionation (B) collected the immediate flow through, even before absorption at 280nm was observed. The number of fractions was reduced, and instead of collecting peaks, several peaks were grouped together. A higher concentration of 1M NaCl eluent was used, instead of 500mM, to remove any proteins that were tightly bound to the column. These fractions were pooled together and tested individually to identify activity. The individual fractions and the pooled fractions did not show any induction. Figure 6.9 shows the bioassay using the pooled fractions, and no induction observed. This suggests, therefore, that the fractionation procedure deactivates the activity of the signal and it is not possible to fractionate using this method.

6.8 Sequence analysis of the 66kDa band

The 66kDa band was N-terminally sequenced at the Aberdeen protein sequencing facility. Sequence analysis of the 14 amino acid sequence was carried out using BLAST, however, no significant homology was found. Recently, analysis was repeated with BLAST short sequence analysis, to look for close or exact matches at the N-terminal end. This search did show similarity to a number of potentially interesting proteins. The BLAST search results are shown in Figure 6.10. (A) shows homology to an acyl CoA oxidase from *Arabidopsis thaliana* which is a 62kDa protein involved in oxidasion of acyl-CoA and is expressed in seeds (Hooks *et al.*, 1996). There is 69%

A



B

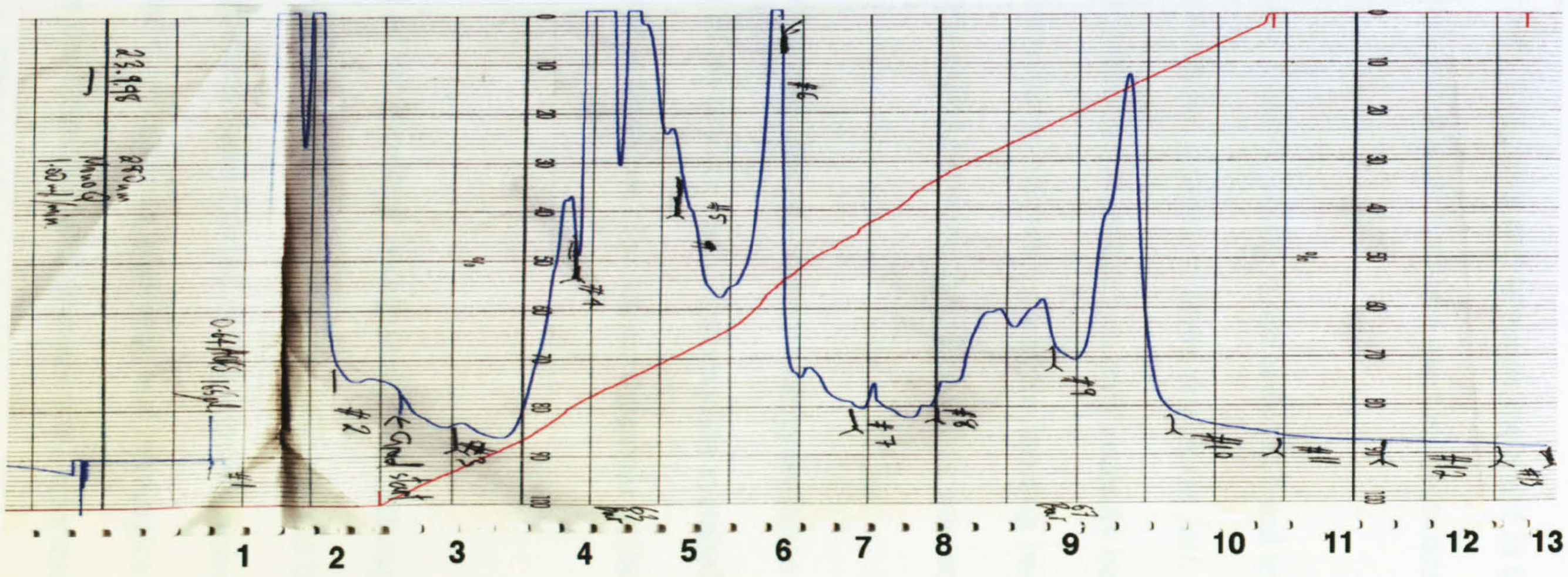


Figure 6.8 The HPLC trace of larval extract. (A) individual peaks were collected. (B) 13 fractions were collected, indicated blow the trace.

sequence identity, however, this is internally and not at the N-terminal. Speculatively, the induced protein in the bioassay, could be a sign of lipid production. The biotinylated protein BCCP was not detected in the bioassay, however, the length of the bioassay may need to be increased for this to be observed. Enzymes and proteins involved in the lipid production pathway, are perhaps a key marker which could be used in the bioassay.

Figure 6.10 (B) shows 100% match across 7 amino acids of our induced band to the N-terminal sequence of a possible outer membrane protein of *Mesorhizobium loti*. Little is known about this outer membrane protein and the sequence has only recently been published (Kaneko *et al.*, 2000). This interesting link with a nodule forming bacteria again suggests the question that similarity exists between the plant-biotic interactions.

The third protein match shown in Figure 6.10 (C) is to a formyltetrahydrofolate deformylase, a 32kDa protein involved in glyoxylate and dicarboxylate metabolism. The molecular mass of this protein suggests that this is not the induced protein in the bioassay.

6.9 Improvement of the bioassay.

Clearly development and improvement of the bioassay is required. In an attempt to find protein markers and cytological characteristics which could be included in the bioassay, protein analysis and cytological investigations were carried out on cynipid inner-gall tissue and are discussed in Chapter 5 and 6. Proteomic, genomic and cytological knowledge of the gall tissue throughout development will provide vital information required for the bioassay's development. DNA probes and antibodies specific to gall

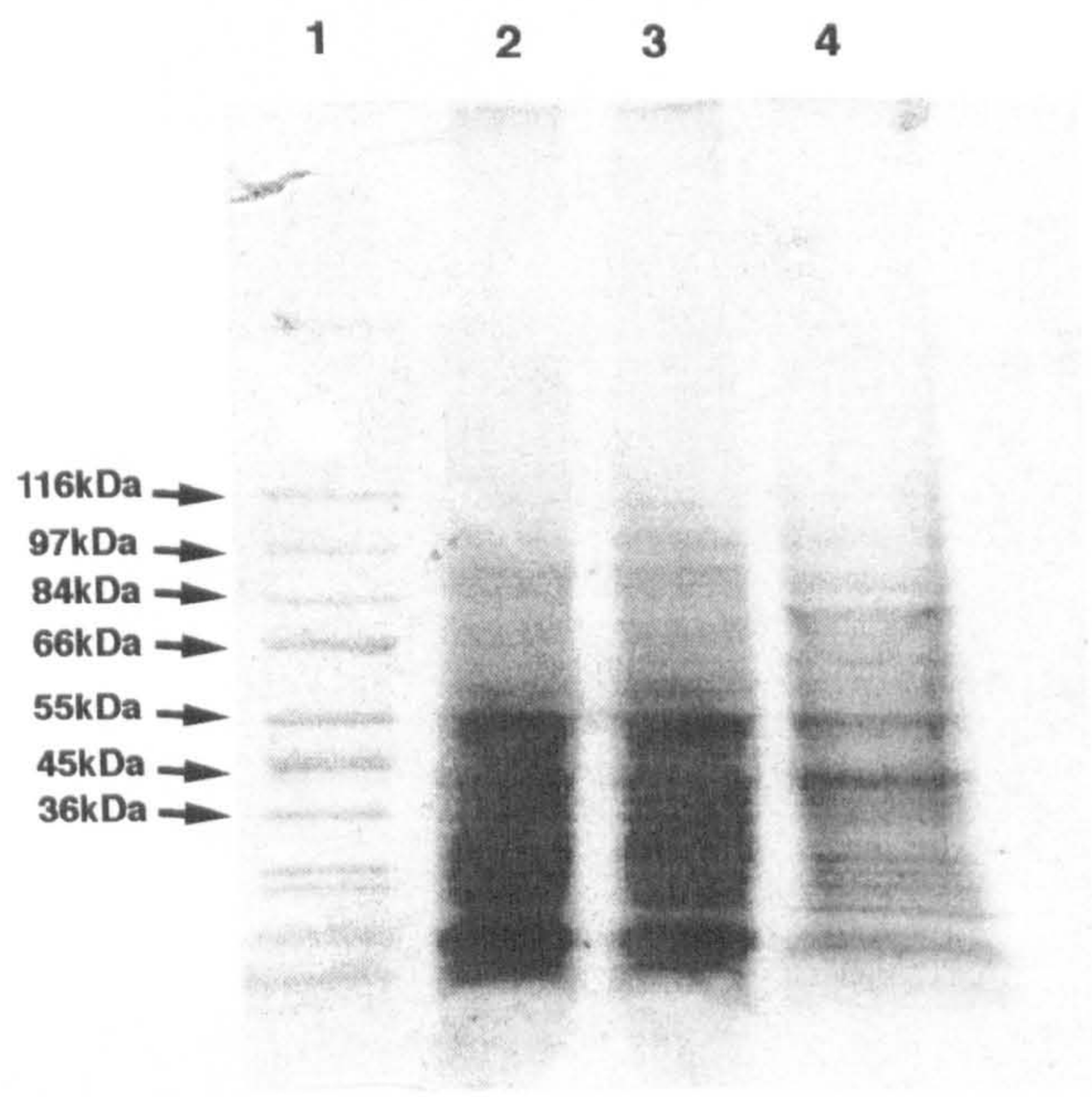


Figure 6.9 A gradient SDS PAGE gel (6.5%-20%) showing bioassay using pooled HPLC fractions of *A.quercuscalicis* larval extract. Lane 1 Wide range molecular weight marker. Lane 2 Bioassay using pooled fractions. Lane 3 Bioassay using total larval extract. Lane 4 *A.quercuscalicis* inner-gall protein extract.

A

```
>gi|3044214|gb|AAC13498.1| (AF057044) acyl-CoA oxidase [Arabidopsis
thaliana]
          Length = 664

 Score = 25.2 bits (52), Expect =    53
 Identities = 9/13 (69%), Positives = 10/13 (76%), Gaps = 2/13 (15%)

Query: 2   YTEVT--LAASKF 12
           YT+VT  LAAS F
Sbjct: 355 YTDVTERLAASDF 367
```

B

```
gi|14021578|dbj|BAB48190.1| (AP002995) outer membrane protein
[Mesorhizobium loti]
          Length = 794

 Score = 23.1 bits (47), Expect =   232
 Identities = 7/7 (100%), Positives = 7/7 (100%)

Query: 8  AASKFLS 14
          AASKFLS
Sbjct: 3  AASKFLS 9
```

C

```
>gi|10581385|gb|AAG20130.1| (AE005091) formyltetrahydrofolate
deformylase; PurU
          [Halobacterium sp. NRC-1]
          Length = 303

 Score = 23.1 bits (47), Expect =   232
 Identities = 6/6 (100%), Positives = 6/6 (100%)

Query: 1  DYTEVT 6
          DYTEVT
Sbjct: 14 DYTEVT 19
```

Figure 6.10. Blast results from N-terminal sequence analysis of induced protein.

formation will provide the specific tool required to test for positive activity of the larval extract. With specific and sensitive markers such as gene or protein expression, the fractionation of the larval extract or secretion can be accurately carried out and the purification of the signals involved will be elucidated. Here the development and optimisation of a possible bioassay for gall formation has been initiated. The molecular information required for this to be taken further are eagerly awaited.

7: Discussion

7.1 Summary of work presented

In this thesis I present research into the molecular mechanism of gall formation which has extended our understanding of the complex molecular mechanisms used by the cynipid larva to reprogramme host development.

7.1.1 Cynipids appear to induce developmental pathways common to secretory cells

The work presented here has shown that cynipids follow a number of developmental pathways to achieve the induction of nutritive cells, ensuring sufficient food for their successful development. The galls analysed show two patterns of development, one involving the induction of enlarged lipid-filled nutritive cells immediately surrounding the larva with an enlarged nucleus and nucleoli, on which they feed throughout the whole of development. The second pathway observed is the induction of enlarged, endoreduplicated, nutritive cells lining an egg-like structure, which breaks down to allow the larva to graze on smaller nutritive cells. The expression of FDH in the species of galls which show the formation of smaller nutritive cells, suggests these cells are under stress whereas the enlarged cells observed in the other pattern of development appear to cope with high metabolic activity by endoreduplication and polytenisation of the chromosomes.

Here I showed the endoreduplication of the whole chromosome, euchromatin and heterochromatin, by fluorescent *in-situ* hybridisation of rDNA loci on the polytene nuclei. Protoplast analysis of the nuclei at different stages of development show an increase in the extent of endoreduplication as development progressed. The induction of the enlarged,

polytene, lipid-filled cells appears to be a mechanism used to cope with the increased metabolic activity demanded of the cell, and is observed in other nutritive secretory cells, such as the suspensor cells and endosperm of seeds.

In chapter 4, expression of putative BCCP, a seed protein, was shown to be expressed in all the inner-gall tissues, at all stages of development. Putative BCCP protein, involved in lipid synthesis, was detected by western blotting analysis and showed the expression of the biotinylated protein is up-regulated in inner-gall tissue, compared to expression levels detected in acorn. This subunit of ACCase is involved in the formation of triacylglycerols, essential for the production of nutrients in the form of lipids. The spatial distribution of the biotinylated protein was visualised by immunohistochemistry. Cy-3 conjugated streptavidin revealed the localisation of the protein and therefore, the production of lipids to be concentrated in the nutritive cells immediately surrounding the larva. In *Andricus quercuscalicis* and *A.fecudator* galls the lipid production is concentrated in the cells lining an egg-like structure, encapsulating the larva for an extended period of time compared to other galls analysed. Once emerged from the structure the cells lining the larva do show putative BCCP expression, although to a lesser extent.

The expression of putative BCCP and the polytenisation of the nuclei in gall cells are typical of nutritive, secretory cells, found in seeds and also in tapetal cells in pollen. The cynipids are clearly using the pathway involved in inducing these nutritive cells seen elsewhere in the plant but not always seen in the organ on which the gall is formed. By manipulating the plant's developmental pathway the cynipid is using the host's capabilities

and inducing these in novel positions for its own advantage.

7.1.2 Inner-gall tissues have species-specific protein signatures

Analysis of inner-gall tissue protein signatures from different gall species showed species-specific signatures and when compared to acorn and leaf, some important similarities were demonstrated. One protein with a molecular mass of 77kDa, appears in all the inner-gall signatures tested and can also be seen in the acorn protein signature. A number of additional bands are common to the seed protein signature and the different inner-gall tissues, although the identification of the 77kDa protein and additional common proteins needs to be determined to confirm this.

7.1.3 What are the larval signals that programme gall formation?

The use of Nod factors in gall formation was the initial hypothesis investigated, and using PCR no *nodC* homologue was found. A 425bp PCR product was amplified by DG42 primers and *nodC* primers and sequence analysis of this did show homology to DG42 and NodC at the primer sites and some internal similarity was present, although not sufficient to suggest *nod* genes are present. The additional PCR products amplified by the DG42 primers on the *A.quercuscalicis* DNA template may show homology, and although not cloned and sequenced here, as they were believed to be non-specific products, sequence analysis of these would confirm if these do show homology or they are true non-specific products. A chitin oligosaccharide synthase, which has diverged from NodC and DG42 may exist in the cynipid genome, and could be used to form chitin oligosaccharides used as signals during the gall formation process. The search for additional *nod* genes, for example *nodA* or *nodB*, using the same PCR technique, would provide clearer evidence as to the possible production of nod factor-like molecules by cynipids. Alternatively, to

approach the question more directly, one could use Nod factors in the bioassay. At present, however, this would not be specific enough to gall formation. Once a gall specific bioassay has been developed and individual genes and proteins related to gall formation have been identified, this direct approach will be possible.

The investigation into the presence of AGPs on the inner-gall tissue cells, compared to non-gall tissues was limited, but did reveal that AGP expression on the inner-gall cells does vary from that of non-gall tissue. Further investigations using additional antibodies and tissue sections of the gall may reveal cell markers specifically expressed at distinct developmental stages of gall development. The analysis using JIM8 antibody in particular would be interesting, as AGPs containing the JIM8 epitope are found on cells with embryonic potential (McCabe *et al.*, 1997). Once embryogenesis is initiated the AGP recognised by JIM8 is only expressed on cells which become the suspensor and maybe have a role in the determination of cell fate (Pennell *at al.*, 1991). AGPs containing the JIM8 epitope indicate the cell's ability to enter embryogenesis, therefore the expression of this AGP on gall cells would be an interesting marker.

7.2 Prospects for future experiments arising directly from this work

7.2.1 Are ENOD or nodule-associated genes expressed in gall tissue?

The expression of ENOD genes in the gall tissue would also be of immense interest.

ENOD genes are expressed in the legume host in response to nod factors, and ENOD40, thought to be involved in the dedifferentiation of cortical cells, has been found to be expressed in root knot nematode galls. The expression of ENOD40 in gall tissue would provide a common and fascinating link to the three plant-biotic interactions. The recent

identification of 22 nodule-associated expressed sequence tags (ESTs) provide additional markers which could be used to investigate similarities between gene and protein expression in cynipid gall and nodule formation (JimÈnez-Zurdo *et al.*, 2000). These novel molecular markers will also aid the elucidation of the genetic control over nodule organogenesis, extending our understanding of biotic-plant interactions. The use of Nod factors, ENOD genes and nodule-associated ESTs in nematode and cynipid gall formation remains to be determined.

7.2.2 Root knot gall gene expression in cynipid gall tissue

Nematode galls show the induction of feeding cells, which undergo endoreduplication as seen in the cynipid feeding cells, although the distinct differentiation of tissue layers is not seen in nematode galls. The nematode-host interaction has been studied in great depth and the whole process, although not understood fully, is clearer than that of cynipid gall formation. Differential gene expression in the feeding cells is in the process of being analysed and the determination of gene expression will provide additional markers which can be investigated in cynipid galls. Similarities in the gene expression in the nematode giant feeding cells and gall nutritive cells may reveal similar manipulation of signalling pathways.

7.2.3 Identification of inner-gall proteins

Sequencing of inner-gall proteins and seed proteins would determine the extent of the similarity between inner-gall protein expression and seed protein expression. Differential protein expression throughout seed development compared to that of inner-gall tissue would determine if only certain stages are induced or if gall formation mimics the whole

of seed formation. The investigation into the expression of seed storage proteins would be interesting and indicate if additional seed characteristics are being used in the gall.

7.2.4 Development of the bioassay

A fundamental tool required for the identification of the active molecules secreted by the larva, to initiate and control gall formation is a bioassay. The bioassay presented in chapter 6 described the preliminary stages in the development of a potentially highly effective assay in the analysis of gall formation signals. To develop the bioassay further, identification of additional inner-gall proteins is required; against which we can use antibodies in western analysis in the bioassay. Western analysis is a sensitive technique which would detect the induction of proteins, not visible when stained by Coomassie Brilliant Blue. The induction of endoreduplication in response to larval extract or secretion could also be used as a possible marker. Sections of exposed tissue could be analysed for increased DNA content using cytophotometric techniques. The optimisation of the assay and the molecular and cytological markers, will enable gall formation and plant signalling molecules to be elucidated.

7.3 Questions to be addressed

7.3.1 How are plant hormones involved?

Gall formation manipulates the distribution of plant hormones to achieve gall growth. The pattern of hormone distribution and the molecular mechanisms used to achieve this manipulation, however, are unknown. The most likely hormones to be involved are auxins and cytokinins. Tryptophan concentration has been shown to be elevated in gall tissue and larvae and it was suggested that the larvae transform this into auxins (Matsui and Torikata

1970). Synergists, which alter the transport and therefore increase local concentration of hormones in certain tissues, have also been suggested as a mechanism for manipulation. Flavanoids are believed to act as synergists and have been identified in gall tissue. Additional auxin synergists have been identified in non-cynipid gall tissue, although the origin of these remains unclear (Sterling, 1952; Brian, 1957; Pilet, 1960; Hori, 1974, 1975,1976; Hori and Miles, 1977; Tandon and Ayra, 1980). The secretion of auxin synergists would achieve manipulation of auxin leading to increased cell division, although the differentiation of tissue layers and the induction of the specialist nutritive cells would probably require a combination of additional signals.

Cytokinins have been isolated from larval extracts and it has have been suggested that these are secreted from the larva (Onkaiva, 1974; Matsui and Torikata, 1970; Matsui *et al.*, 1975). It remains unknown if cytokinins are secreted from the larva, however, their involvement in gall formation is certain. Their involvement is likely to be in the initial and growth phases of gall formation, where increased cell division occurs. Cell division genes such as cyclin genes controlling the G1/S switch and G2/M would be a useful markers to follow the cell division. Comparison to cytokinin distribution would reveal the role played in gall formation.

Brassinosteroids (BR) have been isolated in gall tissue, suggesting that they do have a role in gall formation (Nukima *et al.*, 1983; Nukima *et al.*, 1984). BRs affect cell expansion and cell division although the role of brassinosteroids in plant development is not clearly understood and the response pathways, receptors and overall developmental control in plants, is still to be determined. BRs are relatively newly discovered plant growth

hormones, identified in *Brassica napus* (Grove *et al.*, 1979). Since then, much research has been carried out on this class of growth hormones which have a steroidal structure and structural similarities to the insect hormone ecdysone. BR binds competitively to the insect ecdysteroid receptors demonstrating the similarity between the two hormones (Lehmann *et al.*, 1988). The insect hormone ecdysone is a moulting hormone and binding of BR to the ecdysone receptor inhibits moulting (Lehmann *et al.*, 1988). The binding of the plant growth regulator to an insect receptor demonstrates conservation between signalling molecules of plants and insects which could allow them to communicate. The process of gall formation demonstrates communication between insects and plants, although the method of communication is still to be elucidated. This involvement of brassinosteroids and other plant hormones in gall formation is important, and the further understanding of gall formation and the development of a gall formation bioassay may lead to a model system within which the signalling pathways of these plant hormones can be studied.

Other plant hormones gibberellic acid (GA), abscisic acid (ABA) and jasmonic acid (JA) have not been investigated within gall tissue or in respect to gall formation. Perhaps investigation into the concentration patterns of these hormones would provide a picture as to their involvement and comparison with their role in seed maturation and germination. Perhaps ABA is involved in the maturation stage of gall formation, where the gall desiccates and in some species the pupae overwinter in the desiccated gall.

To identify if the larvae are secreting plant hormone-like signals, gas chromatography mass spectrometry (GC/MS) could be used. GC/MS allows molecules within a small

volume of solution to be separated and identified according to their retention time and searching for selective ions. Databases of known substances can be used to help identify the unknown molecules within the solution. The sensitivity of the technique means the small volume of secretion from the cynipid larva could be used for analysis and the presence of plant hormones or other known active molecules investigated.

7.3.2 Is gall formation related to seed development?

Seed development follows a number of distinct developmental stages from the first asymmetrical cell division to the maturation and desiccation of the seed. Markers indicative of these developmental stages could be used to determine additional similarities between gall formation and seed development. The role of plant hormones in seed development and gall formation is essential. Hormone distribution throughout seed development, and induction of hormone responsive genes within the seed tissue could be used to investigate hormonal distribution patterns of gall formation to determine if galls show similarity to seed development. The expression of hormone responsive genes or those linked to the transport of the hormone in gall tissue at specific stages of gall formation would be ideal markers to follow gall formation. For example, the expression of membrane proteins AUX1 and PIN1, which are influx and efflux carrier proteins involved in the transport of auxin would indicate the temporal and spatial distribution of auxin. PIN1 is normally localised to the basal part of the cell from the early stages of embryogenesis. It is differentially expressed and the expression of PIN1 in gall tissue cells compared to seed development may indicate the direction of auxin transport and any similarities in the manipulation of local auxin concentration. Additional auxin responsive genes may help build a picture of the temporal and spatial distribution of auxin within the

formation of seed and the gall. Auxins have a very important role in embryogenesis, involved in co-ordination of correct cellular patterns; therefore, the elucidation of the manipulation in gall formation would provide important positional information of the developing gall. Similarly cytokinin, brassionosteroid and other hormone-responsive genes will indicate the manipulation of the hormone concentrations, and together show how hormone distribution contribute to the manipulation of normal plant development to achieve gall formation and the extent of similarities to seed development.

7.3.3 Are signalling pathways found in higher model organisms used in gall formation?

In genetics, model organisms are a valuable tool enabling gene function, signalling pathways and developmental processes to be studied. Two higher model organisms *Drosophila melanogaster* and *Caenorhabditis elegans*, which have both been the subject of extensive genetic analysis, mutational analysis and genome sequencing, have provided detailed knowledge of gene regulation and signalling pathways throughout development. The total genomic sequence for both these model organisms has revealed many orthologs in other organism including mice and humans. The two systems have very different patterns of development, *C.elegans* shows a fixed cell lineages, whereas *Drosophila* has a syncitial embryogenic development (Rubin *et al.*, 2000). Despite this there are a number of important shared processes between these models and many other organisms including humans, mice, zebrafish and yeast.

The 120 megbases of *Drosophila* euchromatin has been completely sequenced (Adams *et al.*, 2000). Sequence analysis of the 13,600 genes show 30% have orthologs in the 97 megabases of *C.elegans* genome sequence containing 18,424 genes (Hodgkin *et al.*,

1998). Genetic analysis and mutation based studies have determined the function of many of the genes and the pathways in which they are involved. Three of the major signalling pathways revealed in *Drosophila* are the Hedgehog family of proteins, the Notch transmembrane receptors and Wnt glycoprotein family. The Wnt family of secreted glycoproteins are believed to be involved in cell proliferation, differentiation, polarity and migration (Kühl *et al.*, 2000). The Hedgehog proteins are a family of short and long range signalling molecules which have an important role in developmental patterning and have been identified in several organisms, invertabrate and vertabrate (Hammerschmidt *et al.*, 1997). The active form of the Hedgehog protein is an N-terminal peptide, which is first cleaved from a larger protein and modified by the addition of cholesterol which then becomes attached to the cell surface, where signalling occurs.

The Notch family of proteins are transmembrane receptors which are used in local and intercellular communication controlling morphogenesis, differentiation, proliferation and apoptosis (Artavanis-Tsakonas *et al.*, 1999). Interestingly, Notch is believed to regulate the onset of the endocycle in *Drosophila*, leading to polytene chromosomes (Deng *et al.*, 2001). It is believed that a phosphatase involved in the G2/M switch, known as String, is a target of Notch and when Notch is inactivated then String is expressed and the G2/M switch is blocked, inducing endocycles. The polytene chromosomes formed in the nutritive cells of galls may be regulated by a similar pathway.

In *C.elegan,* more than 40% of the genes have orthologs in *Drosophila* and a number of other organisms and many of the processes found in worm and *Drosophila* are shared. The Wnt glycoprotein and some of the Notch receptor signalling pathways can also be seen in

the nematode, however, the hedgehog protein family are not present. These conserved signalling pathways are used despite the differences in the developmental patterns, suggesting similar pathways may also exist in cynipid gall formation. The developmental genetics determined in *C.elegans* and *Drosophila*, could now be used to investigate if the same conserved developmental signalling pathways are used in gall development.

7.4 Conclusions

The overall objective of the research, to use gall formation as a model system to investigate plant development, has been initiated, although the complexity observed in cynipid gall formation adds difficulty to the system. Internally, however, it is possible to analyse the interruption of normal plant development and investigate the mechanisms used to achieve this. The investigation of manipulation achieved by the cynipid gall wasp presented in this thesis, and the initial development of a bioassay, have provided an insight into the molecular mechanisms used in gall formation. Further development of the bioassay and identification of protein and gene expression in the gall tissue, will enable our understanding to be taken further and determine pathways involved in gall formation and extend our understanding of plant development.

The induction of specialist nutritive cells in gall formation, and the possible use of seed developmental pathways, would provide an opportunity to study these and additional plant developmental steps within the gall. Together with additional seed characteristics such as the expression of seed storage proteins and cytological characteristics such as polytenisation, it will be possible to confirm or reject the hypothesis that seed developmental pathways are used in this fascinating insect-plant interaction.

PAGE NUMBERING AS IN THE ORIGINAL THESIS

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REPROGRAMMING PLANT DEVELOPMENT: TWO APPROACHES TO STUDY THE MOLECULAR MECHANISM OF GALL FORMATION

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Abstract.—Each species of cynipid gall wasps induce the growth of unique galls which are both structurally distinct and anatomically novel structures to their host plant. Although much has been written on the anatomy of cynipid galls, little is known about the molecular mechanisms responsible for gall initiation and growth. Presumably the gall wasps send signals to the host plant to bring about gall formation. Here we present the first results of two approaches to identify these signals.

First, we are trying to identify molecular markers to distinguish between gall and non-gall plant tissue. If such markers can be established, it should also be possible to use marker expression in a bioassay to find and characterise the compounds which serve as signal molecules. We have identified two proteins, expressed in galls of *Diplolepis spinosa* on *Rosa rugosa*, which could prove useful as molecular markers. One protein (90kDa) was found to be exceptionally more abundant in the inner gall tissue, compared to non-gall tissues, such as leaf and stem tissues. The second protein (60kDa) was not detected in the non galled tissues, and appears to be differentially produced in the inner gall tissue, making it an excellent molecular marker.

Nod-factors are signal molecules involved in the interaction/communication between hitrogen fixing bacteria, *Rhizobium* spp., and their legume hosts. To address whether gall wasps use similar signals we are also searching for homologues to nodC, a gene known to be involved in the synthesis of all Nod-factors, in gall wasp genomic DNA. A homologue to nodC in gall wasp DNA suggests that the signal molecules used by cynipids to induce galls might be of a Nod-factor nature. In a polymerase-chain-reaction we used oligo-nucleotide primers designed from NodC and DG42 (a nodC homologue from *Xenopus laevis*) amino acid sequences on gall wasp template DNA. Both sets of primers amplified a fragment of 400bp length from the gall wasp DNA. However, the primers designed from DG42, also amplified two more fragments of 440bp and 1kb length.

INTRODUCTION

Gall formation is arguably the most intimate relationship between herbivorous insects and their host plants, and galls induced by cynipid gall wasps might well be the most sophisticated structures of induced plant growth. Cynipids are able to change the natural growth patterns of their host plants to such an extent that the galls have been described as new plant organs. However, very little is know about the actual mechanisms employed by gall wasps to control the growth of such novel structures.

The shapes and morphologies of cynipid galls vary widely not only between the species of gall-former, but also between generations, where the gall former has more than one generation per year (Dregger-Jauffret and Shorthouse 1992). Mature cynipid galls can be as small as 2-3 millimetres or as large as 10 cm in diameter. Galls of some species house only a single larva whereas others are inhabited by several hundred. Cynipids induce galls on virtually all plant organs and many of them have impressive surface structures, such as spines of variable shapes or glands, which can secrete sugary or otherwise

sticky compounds. Although the gross morphology of cynipid galls may vary, the inner organization of tissues is similar (Rohfritsch 1992). Tissues found in all cynipid galls include a layer of cytoplasmically dense nutritive cells which line the larval chamber, followed by a layer of vacuolate parenchyma, a layer of sclerenchyma, a layer of parenchymatous gall cortex and epidermis (Rohfritsch 1992). Nutritive cells are unique to insect galls and serve the inducers as the sole source of food. Adjoining parenchyma are converted to nutritive cells as the inducer feeds (Bronner 1976). All nutritive and vacuolate parenchyma are considered inner gall tissue in the current study.

Cynipid wasps also control the physiology of gall tissues (Bagatto *et al.* 1996, Harris and Shorthouse 1996). Not only do galls serve as physiological sinks for nutrients and assimilates, but tannins and phenolic compounds, thought to serve as feeding inhbitors for herbivorous insects, are concentrated in the peripheral parenchyma while inner gall tissues have none (Berland and Bernard 1951, Bronner 1976). Starch and glycogen are broken down to their constituents in the inner gall tissue, which results in a measurable gradient from the center to the outside of the gall (Bronner 1976). Transcription and translation rates are increased also in the inner gall tissue, and the cytoplasm of the cells in this tissue are known to be rich in ribosomes and contain large amounts of protein. While the cytology and physiology of cynipid galls has been much studied, the mechanism, i.e., the putative signal molecules or "morphogens", used by the gall wasp to cause all the described changes in the plant tissue is still a mystery.

A number of hypotheses have been put forward concerning the nature of the morphogens, which archive the reprogramming of plant development. These hypotheses suggest the involvement of symbiotic viruses or virus-like-particles, plant hormones, and "other" signal molecules (Cornell 1983, Hori 1992). However, little experimental evidence exists to support any of the hypotheses.

One of the best studied interactions and communication between plants and another organism, is that of *Rhizobium* bacteria and their legume hosts (Denarie *et al.* 1996). Van Brussel *et al.* (1986) showed the significance of Nod-factors for the induction of root-nodules. Chemically Nod-factors are lipo-chito-oligosaccharides, which cause the host plant to develop an infection-thread, through which the rhizobia enter the host plant, and ultimately to develop root nodules in which the bacteria reside. While the Nod-factors produced by different *Rhizobia* species differ in variations of the residual substitutes, they all share the 3-6 monomer long oligosaccharide backbone. NodC was identified as the β-glycosyl-transferase to establish the β-linkages between the glucosamin sugar residues. The fact that NodC produces only oligomeres of a defined length makes it unusual compared to other carbohydrate-synthase-enzymes (Denarie *et al.* 1996). Since the identification of the amino acid sequence for NodC, a number of homologous proteins have been found in a variety of different organisms, e.g., mice, yeast and the frog *Xenopus* laevis (Bulawa 1992, Atkinson and Strong 1992, Spicer *et al.* 1996). It is intriguing that the gene DG42 is only expressed between the midblastula and the neurulation stage of the embryonal development (Semino and Robbins 1995). Like NodC, it appears that the DG42 protein is involved with development. Thus our first approach is to identify homologues to nodC, the gene which codes for the NodC enzyme, in the genomic DNA of *Andricus quercuscalicis* (Burgsdorf).

A more general approach makes no assumptions about the nature of the morphogens. An essential tool to use in the identification of the proposed morphogens would be a bioassay to test extracts made from wasp larvae for gall forming activity. However, since the control of gall-formation could be a complex mechanism, it might be unlikely that it will be possible to induce a gall artificially. It is therefore desirable to have molecular markers in the form of RNA molecules or proteins, which indicate that gall initiation has taken place. Since the cells of the inner gall tissues have been reported to have a dense cytoplasm with high concentrations of proteins, we compare protein extracts from galled and non-galled tissues to identify proteins, which are potential molecular markers. In this second approach we studied the protein contents of inner-gall, outer-gall, and non-galled tissues using galls of *Diplolepis spinosa* (Ashmead) on *Rosa rugosa* Thunb.

The many questions that could be asked in the context of gall formation include: what are the morphogens, how many are there, where do they originate, and what is their effect on the host plant. Here we present the first results from the two approaches outlined above.

METHODS

Protein Extracts From Galled and Non-galled Tissues

Female *D. spinosa* reared from galls collected near Sudbury, Ontario, Canada during April 1995, were exposed to 40 potted *R. rugosa* (Hansa variety) cultivated in growth cabinets at Laurentian University. Galls were harvested and dissected 6 weeks (±1 week) after oviposition. Larvae were removed and inner gall tissue (nutritive tissue and nutritive parenchyma) and outer gall tissue were harvested separately and snap frozen in liquid nitrogen. Simultaneously, non-galled tissues, here leaf tissue and pieces of stem from just below the gall, were

harvested and snap frozen. Protein extracts were made by grinding the tissues under liquid nitrogen using mortar and pestle and subsequent boiling for 5 minutes in SDS-PAGE extraction buffer. Any solid debris was removed by centrifugation. Protein contents were established using the Biorad assay based on Bradford (1976). 20µg total protein of each sample were loaded on a 10 percent SDS-PAGE gel (Laemmli 1970) together with the wide range molecular weight markers from Sigma. The gel was subsequently stained with Coomassie Blue after Dunn (1993).

Polymerase-Chain-Reactions (PCR) in the Search for NodC Homologues

The primers for the PCR reactions were designed as degenerate primers based on the consensus of the aminoacid sequence of 12 known NodC enzymes from different *Rhizobia* species. To achieve a higher specificity with the degenerate primers, the PCR was carried out in two steps, i.e., a nested reaction. In a nested PCR, two different pairs of primers are used whereby the sequences of one pair (the outer primers) are located on the amino acid sequence outside the second pair (the inner primers; table 1). After using the outer primers with template DNA, a second PCR was carried out on the amplified products of the first reaction. Similarily, another set of primers was designed from the consensus sequences between NodC and the DG42 protein, isolated from *X. laevis*. To further increase the efficiency of the PCR, 5 permissive cycles where run at a lower annealing temperature of 42°C followed by 35 cycles with the more stringent annealing temperature of 48°C. The complete PCR program is described in table 2.

The template for the PCR reactions were the cDNAs of nodC and DG42 as controls and genomic DNA of the gall wasp *A. quercuscalicis*. To obtain the gall wasp DNA, larvae were dissected from the autumn galls. The guts of the larvae were removed before the genomic DNA was extracted after a protocol provided by Dr. J. Cook.

Table 1.—*Description of the primers used including the amino acid sequences from NodC and DG42, which were the templates for the design of the primers*

Primer location	Primer name	Amino acid sequence of the template
NodC		
5' end - outer	B5out	YVVDDG
5' end - inner	B5in	NVDSDT
3' end - inner	B3in	MCCCGP
3' end - outer	B3out	QQLRWA
DG42		
5' end - outer	V5out	QVCDSD
5' end - inner	V5in	EMVKLV
3' end - inner	V3in	DDRHLT
3' end - outer	V3out	NQQTRW

Table 2.—*PCR programming steps in the PCR for nodC homologues*

Number of cycles	Temperature	Duration
1	94°C	4min
5	94°C	60sec
	42°C	60sec
	72°C	60sec
35	94°C	60sec
	48°C	60sec
	72°C	60sec
1	72°C	5min

RESULTS

Identifying Proteins in the Inner Gall Tissue

Extracts of total protein, made from leaf-(L), stem-(ST), outer gall-(OG) and inner gall tissues-(IG), show a variety of abundant structural proteins (fig. 1). By comparing the bands in all four lanes, we found that two of the proteins in the extract made from inner gall tissue appear to be particular to this tissue (see arrow heads; fig. 1). At a molecular weight of 60 kDa, the band found in the IG-lane does not appear in any of the other samples and might be specific to inner gall tissues. The protein at 90 kDa is much more abundant in IG, although it is present in all four samples shown here (fig. 1).

Searching for Homologues to NodC and DG42 in Gall Wasps

Degenerate primers, as used here, generally produce some non-specific amplification, so that the PCR-product appears as a smear. However, where homology exists, annealing is more likely and the amplified product will produce a brighter band within the smear. After the first reactions, using the "outer" primers, the PCR products were then used as the template with the appropriate "inner" primers in the second reaction of the nested PCR (fig. 2). The lane marked M shows a 100bp ladders as a size marker, where the top band has the size of 1kb, the next of 900bp and the smallest band visible 200bp. Lane 1 shows the PCR products of the first control reaction with nodC primers and nodC as the template, and lane 3 the second control reaction using DG42 primers and DG42 as the template. In lane 2 and 4 the PCR products of the nodC primers (lane 2) and the DG42 primers (lane 4) are shown, used with the genomic DNA of the gall wasp *A. quercuscalicis* as the template.

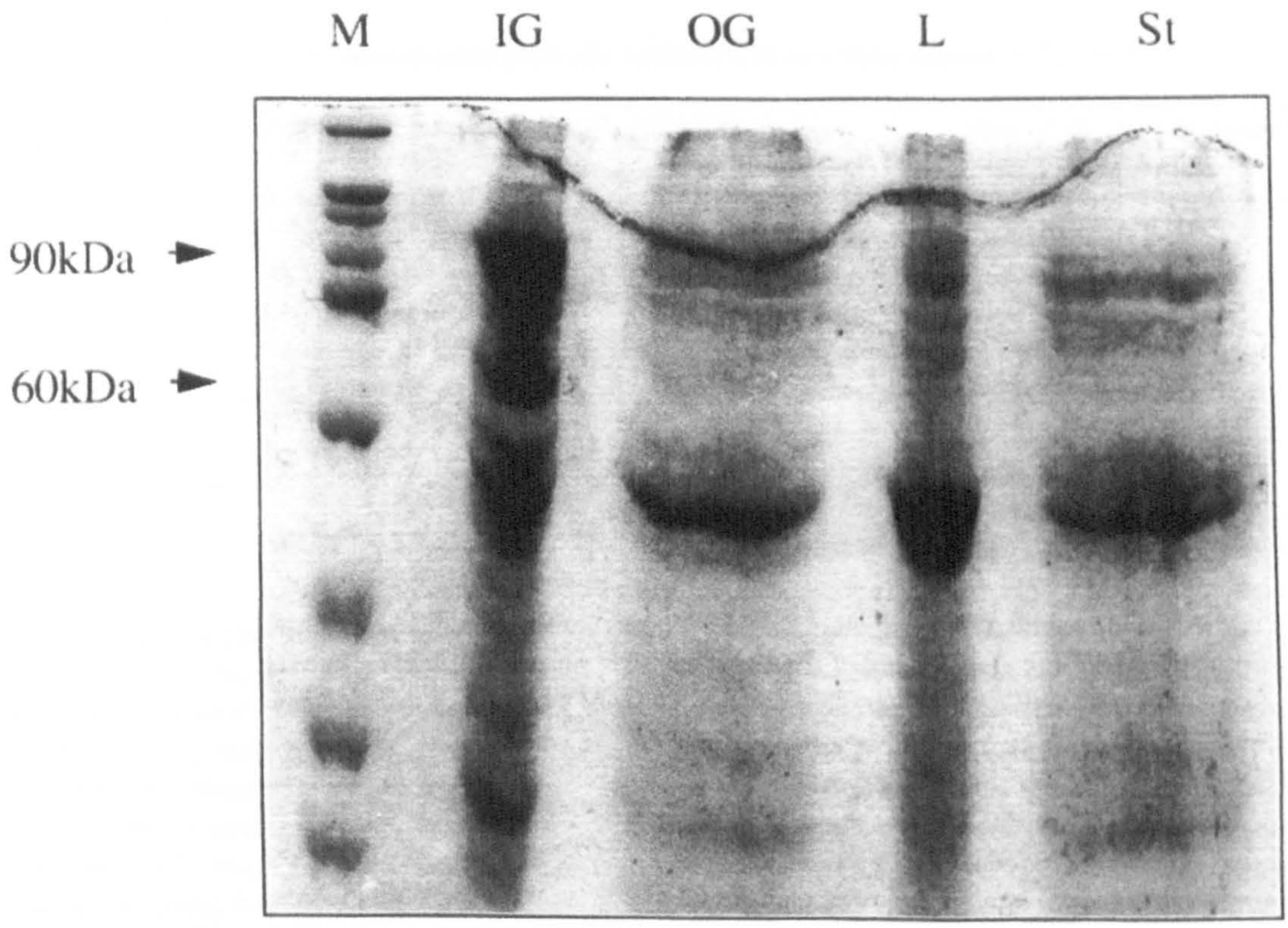

Figure 1.—*SDS-PAGE gel of total protein extracts of inner gall tissue (IG), outer gall tissue (OG), leaf tissue (L) and stem tissue (St). The lane marked M shows the wide range molecular weight marker and the arrow heads point out the two proteins at 60 and 90 kDa, which are candidates to be molecular markers for inner gall tissue.*

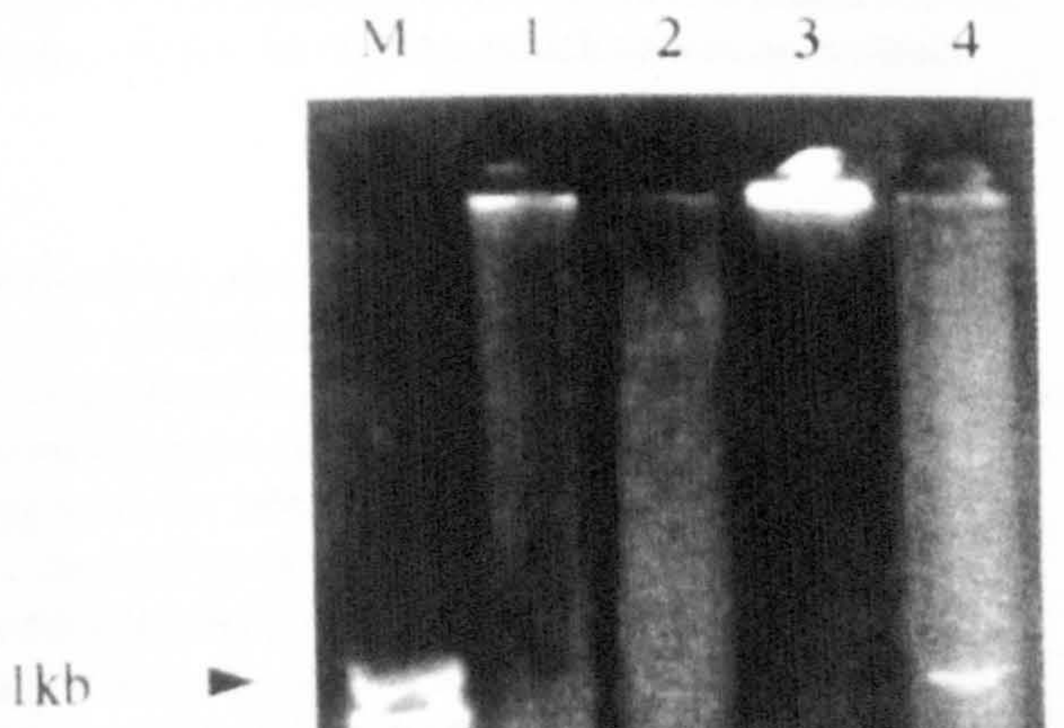

Figure 2.—*Products of the second reaction of the nested PCR. Lane M shows a 100bp ladder as a size marker. The amplified product of nodC primers using nodC cDNA as template is shown in lane 1, nodC primers on gallwasp genomic DNA template in lane 2, DG42 primers on DG42 cDNA template in lane 3 and DG42 primers on gallwasp genomic DNA template in lane 4.*

Table 3.—*Expected and observed fragment sizes from the nested PCR reactions shown in figure 2*

Combinations of primers and template DNA	Expected size of the fragment to be amplified	Size of the bands observed
nodC primers on nodC cDNA template	198bp	440bp, 560bp
DG42 primers on DG42 cDNA template	397bp	390bp
nodC primers on gall wasp genomic DNA template	unknown	400bp
DG42 primers on gall wasp genomic DNA template	unknown	400bp, 440bp, 1kb

The DG42 control reaction produced one band at 390bp, which is the expected size for the DG42 gene (fig. 2; table 3). The expected size for the nodC fragment would have been 198bp (table 3), which we could not detect. It is possible that the nodC fragment was amplified, but was below a detectable concentration against the background of unspecific amplification. Using the gall wasp DNA as the template, both sets of primers amplified a 400bp fragment. The DG42 primers also amplified two larger fragments at 440bp and 1kb. The 400bp fragment, if identical in both reactions, might be the best candidate for a nodC homologue. However, we do not have an expected size for the fragments amplified from the gall wasps genomic DNA, because it might contain introns, unlike the cDNAs, which served as controls.

DISCUSSION

Mechanisms of the initiation and control of organogenesis are currently some of the biggest challenges for developmental biologists. Because during gall formation the morphogens originate outside the developing organism, the interaction between host plant and ceciduous insects will perhaps provide an excellent model system for plant development. However, little is known about these interactions on a molecular level. Here we have shown the early results of two different approaches to gain insight into what the morphogens are and how they affect the host plant.

We were able to amplify DNA fragments from gall wasp genomic DNA using degenerate primers designed from amino acid sequences of NodC and DG42. Sequence analysis will reveal whether the amplified fragments share sequence identity with nodC, which could suggest that the morphogens might be oligosaccharides. Apart from Nod-factors, derivatives of xyloglucan, a polysaccharide which makes up the matrix of cell-walls together with cellulose, have been found to be signalling molecules, which affect cell-cyle and proliferation (Guillen *et al.* 1995). Intriguingly, the biological active derivatives of xyloglucan have the same length as the Nod-factors (3-6 monomers). Longer fragments are not recognized by the cells as signal molecules (Guillen *et al.* 1995). The xyloglucan derivatives represent a plant internal signalling mechanism and it is conceivable that *Rhizobia*, and possibly also cynipid wasps, make use of the plant cell receptors designed for plant internal signalling with oligosaccharides as signal molecules.

A prerequisite to identify and study such morphogens is a bioassay for gall formation. Such a bioassay could be based on morphological changes (Higton and Mabberly 1994). However, gall formation is potentially complex making it unlikely to assemble all components to induce a gall in the absence of a gall former. Here we present the first results of a different approach. By studying the differences of the protein contents between galled and non-galled tissues it is possible to establish gall formation activity without necessarily having to observe morphological changes. The 90 kDa and 60 kDa proteins we identified are good candidates for being molecular markers: (a) they are abundant and in case of the 60 kDa protein seems specific to the inner gall tissue and (b) the inner gall tissue is the first to differentiate during gall formation. The process of tissue differentiation during gall formation has been studied for a variety of different cynipid galls as well as galls formed by other inducers (Hough 1953a, b; Rey 1992; Rohfritsch 1992).

The two proteins offer a number of possibilities to proceed in future studies. We are currently determining the end-sequences of the two proteins, which would allow us to do a database search for homologous proteins with known function. Further research might also involve raising antibodies against the proteins, which could be used to screen tissues of the host plant to investigate where and when in the normal development of the plant these proteins might be produced. Further screening of the inner gall tissues of other cynipid galls

would be of interest to establish whether or not the two proteins are generally produced during gall formation or if they are specific to the interaction between *D. spinosa* and *R. rugosa*. It would also be possible to determine the cDNA sequences for the proteins, which could then be used as probes to identify gall formation activity in RNA expression studies.

Insect-Plant Interaction During Gall Formation

We propose here that oligosaccharide molecules, similar to Nod-factors, might be the type of morphogens used by cynipid wasps to reprogram plant development. It is important to note that the *Rhizobia*—host interaction involves a whole chain of events. The plant produces flavenoids at its root tips, which the bacteria use to orientate themselves towards the hosts. The *Rhizobia* then begin the production of Nod-factors, causing the host-plant to develop a so called infection thread, by which the bacteria can enter the roots. Ultimately the plant will develop nodule primodia into fully developed nodules, where the bacteria will reside. Thus, the recognition of the Nod-factor starts a whole cascade of developmental events. Gene regulation during organogenesis is known to be a complex process. Generally a series of regulatory genes are expressed before any structural genes. Therefore it seems likely that cynipids make use of developmental cascades in the same way that Nod-factors stimulate nodule formation. The inner structure of galls can be compared to that of seeds and it will be interesting to see whether or not the proteins we isolated show any relation to seed tissue. If so, the result would strongly suggest that a "seed developmental cascade" is at least involved in the gall formation process. What these cascades are and how many might be involved in the formation of a gall will be subject of future research.

While the notion that Nod-factor like molecules might be involved in the gall formation process is new to the discussion about active substances, a variety of other compounds have been proposed and are discussed in detail by Hori (1992). Plant hormones, such as auxins and cytokinins, have most often been suspected as morphogens. Other compounds in question are those involved with the regulation of plant hormones, such as the phenol-polyphenol system or indol-acetic-acid (IAA) oxidase. Auxins (IAA, etc.) were found in larvae of *Cynips quercusfolii*, and larvae kept in a solution of tryptophan (a precursor of IAA) seemed to produce IAA (Kaldewey 1965). Kaldewey, however, concluded that the IAA originated in the diet of the larvae. Matsui and Torikata (1970) found only low concentrations of IAA in the larvae of *Dryocosmus kuriphilus*, but high concentrations of tryptophan, suggesting that the secretion of the precursor of the plant growth factor is important to gall formation. Later Matsui *et al.* (1975), studying again extracts of the same cynipid species, suggested cytokinins were the important compounds, while Yokota *et al.* (1973) claimed it was not the plant hormones, although he found an increase of activity by indigenous plant hormones. Thus the role of plant hormones in the process of gall formation is far from clear. In the context here, the question is whether or not plant hormones are morphogens themselves and therefore it would be crucial to show that cynipid larvae are able to produce them. In fast proliferating tissues like those inside a developing gall, plant hormones should play an important role and it would be easy to imagine that a larva would ingest plant produced hormones and even be able to concentrate them in some way in its salivary glands to secret them back to the plant tissue. If this would be the case, however, plant hormones would aid gall formation, but there would still be another signal necessary to initially induce them. Weidner (1957) suggested that cynipid galls are too complex in form, and that cecidogenesis could not simply be explained by plant hormone activity. In fact, we know that plant hormones play the most important role in the formation of "crown galls", induced by *Agrobacterium tumifascians*, and they lack all the morphological complexity so characteristic of cynipid galls.

Other substances suggested as morphogens include RNA (Taylor 1949). RNAs are generally short lived molecules within a cell. To move RNA through intercellular space and into a host plant cell suggests a highly sophisticated system of chaperoning to keep it safe from degradation and RNAase activity. A possible way would be the involvement of a symbiotic virus as suggested by Cornell (1983). Unlike Taylor, who found high concentration of RNA in the saliva of the wasp larvae, Cornell proposed a transfer of viruses with a fluid deposited by the ovipositing female. This would be an intriguing parallel to parasitoid relatives of cynipid wasps, which are known to suppress the immune system of their insect hosts via "viroid particles" which are injected during oviposition (Edson *et al.* 1981, Vinson 1990). Cornell (1983) himself pointed out that gall development stops, if the gall wasp larva is killed, makes it necessary to envisage a mechanism by which the "virus" is only active in the presence of a factor emanating from the gall wasp larva (Magnus 1914, Rohfritsch 1975, Bronner 1976). This scenario would make ecological sense, as it would forge a permanent link in a mutualistic relationship between "viroid" and gall wasps (Cornell 1983). However, there might be another paradox in this kind of relationship. The fact that gall morphologies are species- or even generation-specific would mean that the speciation rates of gall wasp and "viroid" should be synchronized and we are not aware of any proposed mechanism allowing this to occur. Alternatively, one could imagine a situation where the function of a "viroid" would be to facilitate gall formation rather than being the main factor. Thus,

while it is not possible to discard the presence of a mutualistic virus-like organism, we would still suggest that the wasp larvae are the most important source for morphogens.

Rohfritsch (1992) describes the presence of a "cocktail" that bathe the cells which line the larval chamber. This "cocktail" containing salivary enzymes, such as amylases and proteases, from the larva, as well as hydrolases, amino acids, soluble sugars, various metabolites and cell wall fragments from the consumed plant cells. Whether or not the larva actually produces Nod-factor like molecules cannot be decided as yet, but with the presence of the insects chitin and the cell wall fragments swimming in a digestive cocktail there might well be the 3-6 monomer long oligomers present, similar to Nod-factors or the derivatives of xyloglucan (Guillen *et al.* 1995). Hori (1992) pointed out that the microfeeding behavior of the gall wasp larva might determine the ultimate morphology of a gall. While this implies it might be extremely difficult to induce a gall artificially, i.e., without the gall inducing insect, it also suggests that the development of bioassays, as the one proposed here, is all the more important to identify the morphogens, which allow a reprogramming of plant development.

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The protein content of tissues in cynipid galls (Hymenoptera: Cynipidae): Similarities between cynipid galls and seeds

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ABSTRACT

Cynipid galls are examples of induced plant development, where the gall inducer is in control of cell differentiation and morphogenesis of a new plant organ. This study concentrates on the tissues of the larval chamber common to all cynipid galls. The protein content of the inner gall tissue was compared to that of non-gall plant tissues. We investigated three oak and two rose galls and their respective host plants. Total protein signatures of inner gall tissues were different from those of non-gall plant tissues, and among the five galls. N-terminal sequences were obtained for two abundant proteins from the inner gall tissues of *D. spinosa* and *A. quercuscalicis*, which were common to all galls, at 62 and 43 kDa. Database queries suggest the 62 kDa protein to be homologous to a protein disulphide isomerase (PDI), and the 43 kDa protein to be homologous to NAD-dependent formate dehydrogenase (FDH). A naturally biotinylated protein was detected at 33 kDa during Western analyses with streptavidin. Western analyses revealed the presence of the biotinylated protein and PDI in the inner gall tissues of all five galls, while FDH was only detected in *A. quercuscalicis* and *A. fecundator*. PDI was also common to all non-gall tissues, while FDH was not detected in non-gall tissues, and the biotinylated protein was only detected in seeds. The proteins identified in the inner gall tissue suggest that (a) inner gall tissues in some galls are under respiratory stress, and (b) cynipid gall formation might involve the ectopic expression of seed-specific proteins.

Key-words: cynipid galls; gall formation; gall proteins; insect–herbivore interaction; plant development.

INTRODUCTION

Plants interact with non-plant organisms on a number of levels. Metabolic products, such as tannins and phenolics, are produced in the defence against invasive organisms (Feeny 1970; Kuć 1997). In mutualistic relationships, plants can form novel structures as many legumes do when they form nodules to accommodate nitrogen-fixing rhizobia. The development of the nodules results from the regulation of expression of specific genes by the plant in response to signal molecules, so-called nod factors, that originate from the bacteria (for a review see Dénarié *et al.* 1996). In both types of interactions there are well-defined benefits to the host plant.

Another case of induced organogenesis in plants is the development of galls. Gall formation as a life history trait has evolved in many groups of insects, other invertebrates, and micro-organisms (Rohfritsch 1992). A number of hypotheses have been put forward about the selection pressures that favour this trait in herbivorous insects. The microclimate hypothesis points out the beneficial effects of a controlled environment, the nutrition hypothesis suggests that the manipulation of plant tissue optimizes the food resources for the gall-former, and the enemy hypothesis concentrates on the possible protection the gall-former might gain from residence within the gall (Price *et al.* 1987; Stone & Cook 1998). Gall formation surely represents the most intimate form of plant–herbivore interaction, and cynipid galls (Hymenoptera: Cynipidae) are arguably the most complex structures of all insect-induced galls. The morphologies of cynipid galls are species-specific to the inducing cynipid, and, where the gall-former has more than one generation per year, even generation-specific (Dregger-Jauffret & Shorthouse 1992). Mature cynipid galls may be as small as 2 or 3 mm or as large as 10 cm in diameter. Galls of some species house only a single larva whereas several hundred inhabit others. Cynipids induce galls on virtually all plant organs, although most species are very specific about the location of their galls. Many of them have impressive surface structures, such as spines of variable shapes, or glands that secrete sugary or otherwise sticky compounds (Stone & Cook 1998). Although the overall morphology of cynipid galls may vary, the inner organization of tissues is similar (Rohfritsch 1992). Tissues found in all cynipid galls include layers of cytoplasmically dense nutritive cells that line the larval chamber, bounded externally by a layer of vacuolate parenchyma. In most cases, these two tissues are encapsulated by a layer of sclerenchyma that can be seen as the boundary layer of the larval chamber. Nutritive cells are unique to insect galls and serve the gall inducer as the

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sole source of food (Rohfritsch 1992). The adjoining cells of vacuolate parenchyma are converted to nutritive cells as the inducer grows and grazes on the tissue (Bronner 1992).

Cynipid wasps also control the physiology of gall tissues (Bronner 1977, 1992; Bayer 1992; Bagatto *et al.* 1996). Not only are galls physiological sinks for nutrients and assimilates, but tannins and phenolic compounds, thought to serve as feeding inhibitors for herbivorous insects, are concentrated in the peripheral parenchyma (distal from the larval chamber), while inner gall tissue contains none (Berland & Bernard 1951; Bronner 1992). Starch and glycogen are broken down to their constituent compounds in the inner gall tissue, which results in a measurable gradient from the centre to the outside of the gall (Bronner 1992). Gene transcription and translation rates are increased in the nutritive tissue, and the cytoplasm of these cells is known to be rich in ribosomes and to contain large amounts of protein.

The relationship between plant and gall-former is thought to be parasitic, since there are no known benefits to the host plant in forming a gall. Thus, the gall-inducer must have evolved the means to over-ride normal plant development. In spite of the considerable amounts of data that have been accumulated about the manipulation of plant tissue by the cynipid gall-inducers, it is neither known what these means are, nor what degree of control they exert over the development of their host plant (Cornell 1983; Hartley & Lawton 1992; Hori 1992; Hartley 1998; Schönrogge *et al.* 1998).

To identify such a mechanism would be of immense interest for plant development studies, because the signal molecules used by the insects to induce galls (an external source) seem likely to be either identical or at least to mimic signal molecules the host plant uses during its development. Furthermore, understanding gall formation is also important in the population dynamics of these species to measure resource availability, and one can envisage future studies exploiting any demonstrated mechanism in manipulative experiments.

Here we investigate quantitative and qualitative aspects of the protein content of inner gall tissues of three cynipid galls induced on oak and two induced on roses: *Andricus fecundator, A. kollari, A. quercuscalicis*, and *Diplolepis rosae* and *D. spinosa*, respectively. Comparison of inner gall tissues to non-gall tissues of the host plants *Quercus robur*; *Rosa canina* and *Rosa rugosa* allow us to ask questions about common characteristics and/or differences among galls, the degree of control exerted by the gall-former and about the relationship between gall tissue and 'normal' plant tissues.

METHODS

Materials

In this study, the lining of the larval chamber that is comprised of nutritive and vacuolate parenchyma is referred to as inner gall tissue. To extract proteins, inner gall tissues were dissected from five cynipid galls on English oak (*Quercus robur*): *Andricus fecundator, A. kollari, A. quercuscalicis* (all autumn galls containing the agamic generation), and two rose galls: *Diplolepis rosae* (on *Rosa canina*) and *D. spinosa* (on *R. rugosa*). From the host plants we collected leaf, stem and seed tissues. All tissues were snap-frozen in liquid nitrogen and stored at – 70 °C until further processing.

Protein extraction and determination of protein concentrations

All tissues were ground with mortar and pestle under liquid nitrogen. Proteins were then extracted using an extraction buffer after Shimoni *et al.* (1995). Any solid debris was removed by centrifugation (16 000 *g* for 15 min). Protein contents were measured using the Bio-Rad protein assay (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) based on the method of Bradford (1976) according to the instructions of the supplier.

For SDS–PAGE analyses, SDS sample buffer (Dunn 1993) was added to the ground tissues and the sample boiled for 15 min. Aliquots of 20 μg total protein of each sample were loaded on a 6·5–20% SDS–PAGE gradient gel (Laemmli 1970) together with wide-range molecular weight markers from Sigma-Aldrich Company Ltd (Poole, UK). The gels were subsequently stained with Coomassie blue according to the method described by Dunn (1993).

Sequence analyses

Protein sequences were provided by the protein facility at Aberdeen University. The sequences obtained were then matched against database records using the FASTA search engine (Pearson & Lipman 1988).

Western analyses

For all Western analyses, SDS–PAGE gels were run as described above and electroblotted onto Hybond C membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). The membranes were blocked overnight at 4 °C and washed in PBST (per litre: NaCl 8 g, KH_2PO_4 0·2 g, Na_2HPO_4 1·15 g, KCl 0·2 g, Tween-20 20 mL) before further incubations. Two types of Western analyses were carried out. (1) To detect naturally biotinylated proteins, the membrane was incubated for 30 min at room temperature with streptavidin/horseradish peroxidase. (2) Two antibodies (both raised in rabbits) were used to detect specific proteins that were indicated during the sequence analysis. Anti-protein disulphide isomerase antibodies and anti-formate dehydrogenase antibodies were kindly provided by Dr R. A. Dixon (The Samuel Roberts Noble Foundation Inc., Ardmore, Oklahoma, USA) and Dr R. Remy (Université Paris Sud, Centre D'Orsay, Institut de Biotechnologie des Plantes, Orsay, France), respectively. To detect these specific proteins, the membranes were incubated with the primary antibody for 1 h at room temperature, washed, and incubated for 1 h at room temperature with a goat anti-rabbit/horseradish peroxidase antibody (Sigma). After further

washes, an ECL (enhanced chemo-luminescence) detection kit (Amersham) was used according to the supplier's instructions.

RESULTS

Comparison of the protein contents of inner gall tissues of different cynipid galls and between inner gall and non-gall tissues

Total protein was extracted from 100 mg of the frozen tissue. Of the non-gall tissues of both host plants, *R. canina* and *Q. robur*, the seed tissues contained the highest concentrations of protein (Table 1). Among the inner gall tissues, those from *A. quercuscalicis* showed the highest protein concentration, more than twice that of acorns (Table 1). While the protein concentration in the inner gall tissue of *A. fecundator* galls is roughly comparable to acorns, inner gall tissue from *A. kollari* galls contained less protein than any of the non-gall tissues. However, most striking was the low protein concentration of the inner gall tissues from the rose galls *D. rosae* and *D. spinosa*, which was less than half that of the non-gall tissues on *R. canina*.

The protein signatures of the inner gall tissues of *A. quercuscalicis* and *D. spinosa* on SDS–PAGE are distinct from those of the non-gall tissues of *Q. robur* and *R. rugosa* (host to *D. spinosa*), respectively (Fig. 1a,b). The strongest bands in non-gall tissues were found at 55 kDa in leaves (most likely the larger subunit of Rubisco), and at 18 and 35 kDa in seeds (most likely the α-and β-chains of the 11–12S globulins, common seed storage proteins; Fig. 1a,b).

A comparison of the protein signatures of the inner gall tissues of *A. quercuscalicis* and *D. spinosa* shows that some abundant proteins are specific to either species (e.g. the largest ones in both samples at 74 kDa in *A. quercuscalicis* and at 84 kDa in *D. spinosa*), while both samples have others in common (at 55, 43 and 35 kDa; Fig. 1c).

Sequence analyses

The N-terminal sequences were obtained from two proteins: (a) 15 amino acids of a 43 kDa protein from the inner gall tissues of the agamic galls of *A. quercuscalicis* (AqIg43), and (b) 11 amino acids of a 62 kDa protein from the inner gall tissue of *D. spinosa* (DsIg62). The best alignments against database records, using a FASTA search, are shown in Fig. 2. Thirteen out of 15 amino acids (86·7%; plus one conservative substitution) of AqIg43 were identical to formate dehydrogenase (FDH) isolated from *Solanum tuberosum* (Fig. 3a). FDH from *S. tuberosum* has a molecular weight of 41·8 kDa, which is close to that of AqIg43.

Eight out of 11 amino acids of DsIg62 matched the sequence of protein disulphide isomerase (PDI) isolated from *Medicago sativa* (Fig. 3b). PDI is known as a homodimer with a molecular weight of 58·1 kDa, which is similar to DsIg62.

Western analysis between galls

Using antibodies raised against PDI in *M. sativa* and against FDH in *S. tuberosum*, we tested protein samples of five galls, *A. fecundator*, *A. kollari*, *A. quercuscalicis*, *D. rosae* and *D. spinosa* for the presence of the two proteins. Both antibodies cross-reacted with proteins in at least some of the samples and gave positive signals at the expected molecular weights. In earlier experiments and with the use of streptavidin (specific to biotin as a ligand), we also detected a naturally biotinylated protein at 33 kDa. Western analyses using only streptavidin were also included in subsequent Western analyses.

The naturally biotinylated protein and PDI were found to be common to the inner gall tissues of all five cynipid galls tested here (Fig. 3a,c). PDI showed three bands instead of the expected one (as a homodimer). However, different forms of PDI with slightly different sizes are known from database records. FDH could only be detected in the inner gall tissue of *A. quercuscalicis*, from which the original sequence was obtained, and in the galls of *A. fecundator* (Fig. 3b). Neither the sample from *A. kollari*, nor those from the two rose galls, *D. rosae* and *D. spinosa*, showed any signal in response to this antibody.

Western analysis of gall and non-gall tissues

We tested non-gall tissues of the host plant for the expression of FDH, PDI and the naturally biotinylated protein. Most intriguingly, the biotinylated protein was only detected in tissues of acorns, but not in either leaf or stem tissue (Fig. 4a). PDI was found to be present in all the non-gall tissues. The presence of PDI seems not to be related to

Nutritive tissues Cynipid species	Protein concentration (μg/100 mg tissue)	Non-gall tissues Tissue type	Protein concentration (μg/100 mg tissue)
On oak		*Rosa* leaf	296·44
A. quercuscalicis	2564·56	*Rosa* stem	284·51
A. fecundator	877·06	*Rosa* seed	314·03
A. kollari	134·8		
On rose		Quercus leaf	234·47
D. rosae	91·99	*Quercus* stem	322·19
D. spinosa	95·16	*Quercus* seed	1046·763

Table 1. Protein concentrations in samples of 100 mg of frozen inner gall and non-gall tissues

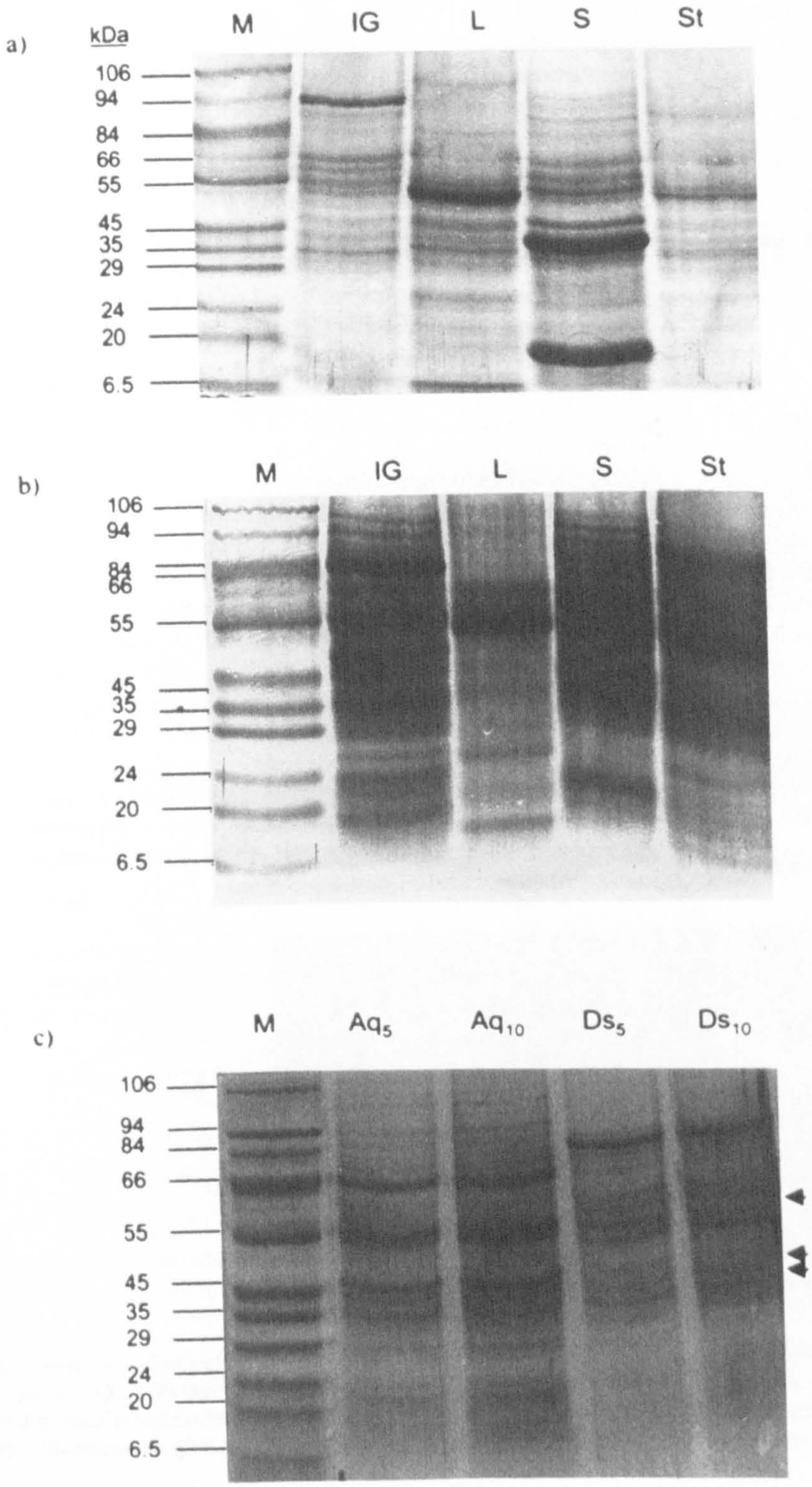

Figure 1. The protein signatures of (a) the inner gall tissues of *D. spinosa* compared to the non-gall tissues of *R. rugosa*, (b) the inner gall tissues of *A. quercuscalicis* compared to the non-gall tissues from *Q. robur*, and (c) the inner gall tissues of *A. quercuscalicis* compared to those of *D. spinosa* on a 6·5–20% SDS–PAGE gradient gel. Abbreviations: M, molecular weight marker; L, leaf; ST, stem; S, seed; IG, inner gall tissue from *A. quercuscalicis* and *D. spinosa*; Aq_5, Aq_{10}, Ds_5 and Ds_{10} are protein samples of the inner gall tissue of *A. quercuscalicis* and *D. spinosa* of which 5 and 10 μL were loaded to visualize abundant and rarer proteins (the arrows on the right indicate bands that both sources of inner gall tissue have in common).

```
AqIg43    LHASPGSKKIVGVFY          DsIg62    DEADXKEFVLTXD
          :.:::: ::::::::                    : :::::: :
FDH       LQASPGPKKIVGVFY          PDI       SSTDAKEFVLTLD
                  30
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Figure 2. Alignments of AqIg43 to the FDH sequence from *S. tuberosum* and DsIg62 to the PDI sequence from *M. sativa*.

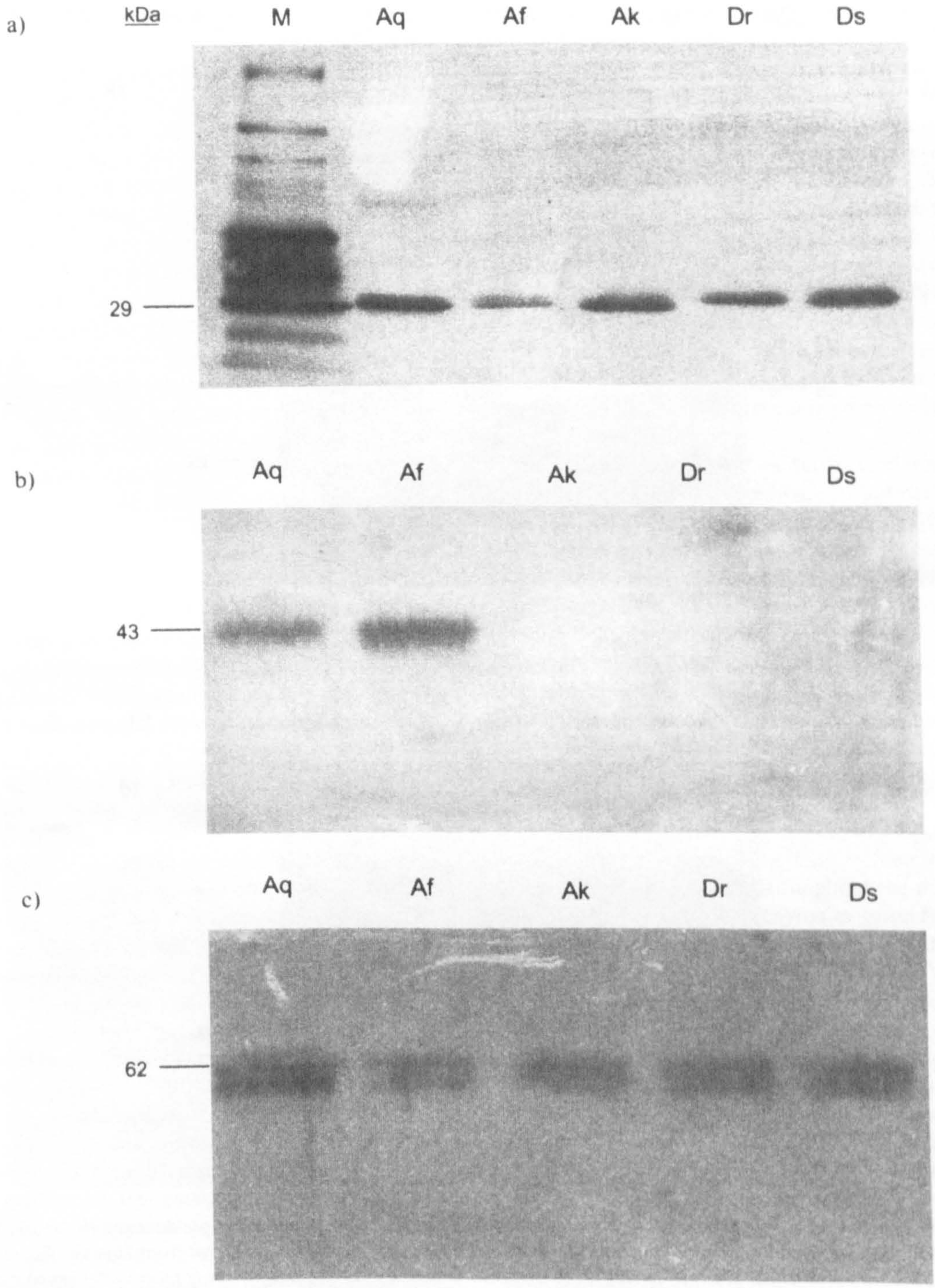

Figure 3. Western analyses of inner gall tissues of the five cynipid galls on a 6·5–20% SDS–PAGE gradient gel: Aq, *A. quercuscalicis*; Af, *A. fecundator*; Ak, *A. kollari*; Dr, *D. rosae*; Ds, *D. spinosa*, incubated with (a) streptavidin, (b) anti-FDH and (c) anti-PDI. The biotinylated molecular weight marker seen in (a) could not be used for (b) and (c), since that would necessitate incubation with streptavidin. Molecular weights for (b) and (c) were established using a rainbow marker (Sigma) that is not visible on the film.

the fact that galls are somewhat specialized plant tissues and is certainly not specific to inner gall tissue (results not shown). In contrast, FDH was detected only in the inner gall tissue of *A. quercuscalicis*, whereas none of the non-gall tissues of *Q. robur* contained this protein (Fig. 4b). While the inner gall tissue of *A. quercuscalicis* provided a positive control to test the non-gall oak tissues, there was no positive control for the protein samples from rose. Since FDH was not detected in the inner gall tissues of either of the rose galls, it was impossible to distinguish whether FDH is not expressed or whether the FDH antibody does not cross-react with the rose protein.

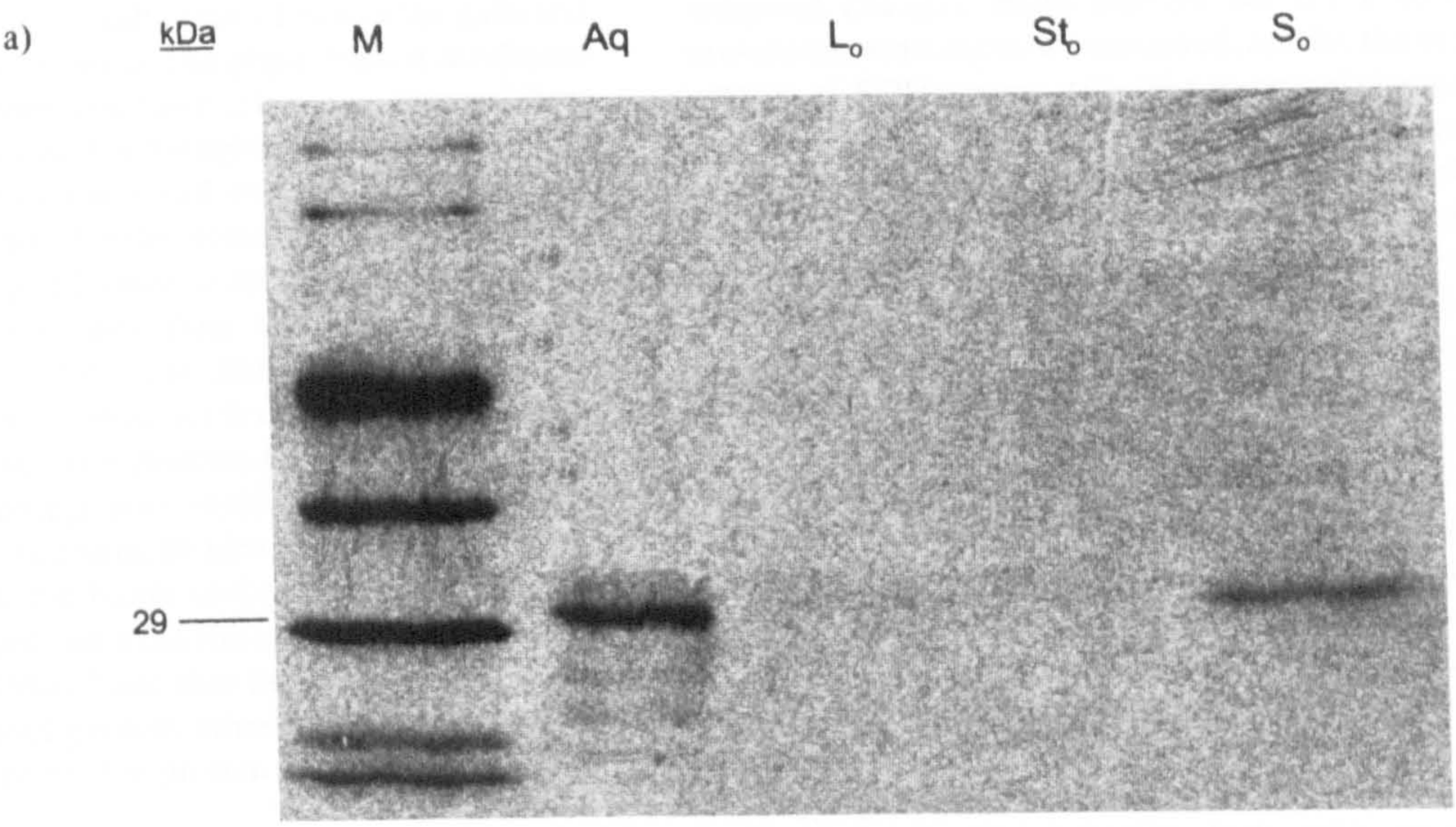

Figure 4. Western analyses of inner gall tissues in comparison with non-gall tissues using (a) streptavidin and (b) anti-FDH. Abbreviations: M, molecular weight marker; L_o, L_r, leaf; St_o, St_r, stem; S_o, S_r, seed; Dr, inner gall tissue from *D. rosae*; Aq, inner gall tissue from *A. quercuscalicis*. The subscripts 'o' and 'r' indicate oak and rose tissues. Molecular weights for (b) were established using a rainbow marker (Sigma) that is not visible on the film.

DISCUSSION

Gall tissues have been shown to be different from 'normal' host plant in their cytology, lipid physiology and carotenoid composition (Czeczuga 1977; Bayer 1992; Bronner 1992). Based on comparisons between gall and non-gall tissues of the concentrations of nitrogen and water-soluble carbohydrates, as well as the activity of phenylalanine ammonia lyase, it has been suggested that the gall-inducer is in control of the nutrient supply and the synthesis of defensive compounds (Hartley & Lawton 1992; Hartley 1998).

Here the content of total protein concentrations and the expression of three specific proteins in the inner gall tissue of five different cynipid galls and in non-gall plant tissues was studied. The expression of the naturally biotinylated protein and PDI was a characteristic common to all galls. While PDI was also present in all non-gall tissues, the expression of the naturally biotinylated protein in non-gall

tissues was specific to seed tissues. By contrast, FDH was detected only in the inner gall tissue of two of the galls and in none of the non-gall tissues. The physiological conditions in the inner gall tissues, and their relation to 'normal' host plant tissues, are discussed in the light of the expression patterns of the three proteins in gall and non-gall tissues and their known functions. Finally, some inferences as to the likely nature of the gall formation mechanism are possible.

PDI and FDH have described functions in *Medicago sativa* and *Solanum tuberosum* (Shorrosh & Dixon 1992; Hourton-Cabassa *et al.* 1998). As for the naturally biotinylated protein, only six such proteins have been recorded to date in plants (Elborough *et al.* 1996). The molecular weight of the protein detected here, 29 kDa, suggests that it might be a homologue of the biotin carboxylase carrier protein (BCCP) that was isolated from the seeds of *Brassica napus* (Elborough *et al.* 1996). Note that the only sources for the naturally biotinylated protein other than gall tissue were seeds. We will refer to this protein as a 'putative BCCP' from here on.

DsIg62, now found to be a homologue to PDI from the inner gall tissue of *D. spinosa,* was previously suggested to be specific to inner gall tissues (Schönrogge *et al.* 1998). Although it has been stated that PDI might have functions in the context of induced defences in plants, it is also known to be a general housekeeping enzyme involved in protein packing and repair (Herman 1994; Shimoni *et al.* 1995). Therefore, it is not surprising that the Western analysis showed that PDI is in fact not specific to inner gall tissues, but is also expressed in all non-gall tissues that were tested.

FDH, however, was specific to the inner gall tissues of *A. quercuscalicis* and *A. fecundator* and was not detected in any of the non-gall tissues. NAD-dependent formate dehydrogenase, the antigen to the antibody, is well known from bacteria, but was only recently also recorded in *Solanum tuberosum* (Hourton-Cabassa *et al.* 1998). FDH is inducible in the leaves of *S. tuberosum* by the exclusion of light for at least 16 h (Hourton-Cabassa *et al.* 1998). By that time, fermentation processes supplement the respiratory processes in the cells. Thus, the expression of FDH is an indicator of respiratory stress in the tissues where it is expressed. That inner gall tissues might suffer respiratory stress was also indicated by studies of the configuration of membrane lipids (Bayer 1992). The expression of FDH in the inner gall tissues of *A. quercuscalicis* and *A. fecundator* correlates with the concentrations of total proteins found, indicating that the accelerated protein biosynthesis might be responsible for the respiratory stress. The inner gall tissue is composed of two tissues: the nutritive tissue and the nutritive parenchyma (Rohfritsch 1992). The nutritive tissue is reported to have exceptionally high concentrations of proteins (Bronner 1992). While Bronner (1977, 1992) reports that the number of layers of nutritive tissues can vary between different galls, it is also known that, for example in the galls of *Diplolepis nodulosa*, the nutritive tissue increases in depth from 2–3 layers to 6–7 layers during maturation (Brooks & Shorthouse 1997). It seems likely that the variability in the ratio of nutritive tissue to nutritive parenchyma, whether species-specific or because of temporal changes, might account for the differences in protein concentrations we measured, and for the expression pattern of FDH among galls. The temporal changes in the protein concentrations and the expression of the particular proteins we studied here will need further research.

The BCCP described from the seeds of *B. napus* is a subunit of a class II CoA-carboxylase that is involved in the synthesis of unsaturated lipids (Elborough *et al.* 1996). Nutritive tissues of cynipid galls are known to contain large numbers of liposomes and generally high concentrations of glycerid lipids, which lends further support to the suggestion that the biotinylated protein observed here is a BCCP (Bayer 1992; Bronner 1992). More evidence and particularly the sequence of the protein from the inner gall tissues would be desirable.

The presence of a BCCP as well as the large numbers of liposomes links the nutritive tissue to 'normal' plant tissues with such characteristics (Bronner 1992). BCCP was isolated from the seeds of *B. napus,* where suspensor cells are known to contain particularly high lipid concentrations (Malik *et al.* 1976; Elborough *et al.* 1996). The function of these cells is to nurture the plant embryo via secretions. Suspensor cells have an equivalent in tapetum cells, which have similar characteristics, but nurture developing pollen grains (Wu *et al.* 1997). Tapetum cells eventually collapse into a sticky coat around the pollen grain.

The feeding mode of gall wasp larvae is not clear yet. However, while the more mature larvae are believed to use their mandibles to tear the plant cells open, this seems unlikely for younger larvae, because their mandibles are too weak (Bronner 1992). Throughout the development of the larvae, nutritive parenchyma is turned into nutritive tissue, and it appears that cell autolysis is the fate of this tissue (Bronner 1977, 1992). Bronner (1992) suggests that autolysis would be induced by the feeding larva through its physical action or by the secretion of proteolytic enzymes. If the cynipids induce a 'tapetum developmental pathway' as part of the gall-formation process, however, it might be possible that the collapse of these cells is pre-programmed into the tissue.

Previous suggestions that galls are somehow linked to seeds or fruits were based purely on observations of similarities in the general appearance of galls and seeds, their organization of tissues, and their functions ('to protect what is inside', Jenkins & Mabberly 1992). Here, for the first time, evidence is presented that links gall tissue to 'normal' plant tissues that are otherwise involved in seed or pollen development.

The similarity of inner gall tissues to suspensor or tapetum cells suggests that the basis for gall formation by cynipid wasps might be the ability to switch on parts of a 'seed development pathway'. Thus, it would not be necessary for the gall wasp to control every detail of the gall development. The nature of the signals that either cause the differentiation of inner gall tissue and/or that lead to the variable, complex and sometimes spectacular structures that make up the overall gall morphologies has still to be

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identified. Understanding the similarities and differences between gall and non-gall tissue should allow the development of techniques to isolate and identify these morphogens.

ACKNOWLEDGMENTS

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105 Soybean Seed Peroxidase Deters Insect Feeding

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Seed coat peroxidase activity in soybeans is controlled by a single dominant gene *Ep*. Soybean plants that carry the *Ep* allele accumulate an anionic peroxidase enzyme in the hourglass cells of the seed coat, whereas *epep* plants do not. This peroxidase is the most abundant soluble protein in seed coats of *Ep* plants, but there is no difference in structure or lignification of the seed coat that is associated with the *Ep* peroxidase. The contents of the hourglass cells including the peroxidase are released during seed imbibition. To determine whether peroxidase accumulation in soybean seed coats may affect feeding by insects, *Delia platura* (seed corn maggot) larvae were given a choice to feed on either high (*EpEp*) or low (*epep*) peroxidase seed types. Imbibed seeds were buried in moist sand in petri plates and larvae were released on the surface. The larvae favoured low peroxidase seeds by a 3:1 margin. When given no choice, similar numbers of larvae feed and survive to pupation on high or low peroxidase seeds. These results demonstrate that *Delia platura* larvae have a clear preference for low peroxidase seeds when provided with a choice. However, high peroxidase seeds do not appear to be severely toxic to the insects since larvae could successfully rely on *EpEp* seeds as food source. We conclude that the peroxidase released from the seed coats deters feeding and causes the larvae to search for other more appealing sources of food. The peroxidase enzyme may directly inhibit feeding, but we feel it is more likely that the enzyme generates active molecules from phenolic precursors that are co-released into the surrounding micro-environment during seed imbibition.

106 The substrate specificity of the wound-induced leucine aminopeptidase (LAP-A) of tomato.

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Leucine aminopeptidases are hexameric metallopeptidases found in animals, plants and prokaryotes. Tomatoes express two classes of leucine aminopeptidase (LAP-A and LAP-N). LAP-A is wound induced, while LAP-N accumulates constitutively. LAP-A is synthesized as a 60-kDa preprotein. Cell fractionation studies indicate that LAP-A accumulates in both the cytosol and the chloroplast. LAP-A is a hexameric enzyme composed solely of acidic LAP subunits. The mature LAP-A protein was over-expressed in *E. coli*. The substrate specificity of the FPLC-purified LAP-A was tested using amino acyl-*p*-nitroanilide and –*b*-naphthylamide substrates. The tomato LAP-A preferentially hydrolyzed substrates with N-terminal Leu, Arg and Met. The specificity of the tomato LAP-A was compared to the *E. coli* and porcine LAP homologues. The tomato LAP-A was also over-expressed as a His_6-LAP-A fusion protein. The His_6-LAP-A subunits assembled into a hexameric enzyme in *E. coli* and was purified to near homogeneity using a Ni-affinity column. The FLPC-purified LAP-A and His_6-LAP-A enzymes had similar K_ms, k_{cat}s, and V_{max}s. The ability of the tomato His_6-LAP-A, porcine LAP, and *E. coli* PepA (LAP) to hydrolyze 60 dipeptide and 7 tripeptide substrate were tested. The LAPs preferentially hydrolyzed substrates with N-terminal (P1) aliphatic residues, Arg, and Met. Substrates with P1 Gly, Asp or Glu were hydrolyzed inefficiently. The nature of the P1' residue had a profound impact on hydrolysis rates. Substantial differences in the activities of the animal, plant and prokaryotic LAPs were established.

107 Plant Galls: Unravelling the Mystery of How Insects Reprogramme Plant Development

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A variety of organisms can reprogramme plant development to produce galls. Some of the most complex and elaborate of all galls are formed by cynipid gall wasps, although the signalling mechanism used by cynipid wasps to induce and control gall formation is not understood. The larvae seem to control the proliferation and differentiation of host plant tissue to form many layers of different cell types, comprising the gall structure. One of the first gall tissues formed in the host plant is the nutritive tissue. This tissue is common to all cynipid galls and provides nutrients for the developing larvae.
Characterisation of the unique biochemical and physiological features of these nutritive cells have provided our focus to study what signalling processes are used by the larvae to control plant development. We have carried out SDS-PAGE analysis of gall tissue to reveal inner-gall-specific proteins. A 29kD naturally biotinylated protein, a putative biotin carboxylase carrier protein (BCCP), which is normally found in seeds, is expressed in all 5 inner-gall tissue tested. BCCP is involved in the synthesis of lipids, which are abundant in the inner-gall tissue. Formate dehydrogenase (FDH), a protein expressed in stressed tissue, has also been found in 2 species of the 5 inner-gall tissues tested.
Cytological studies also reveal morphological differences between gall and non-gall tissues. This has shown interesting variations in chromosome structure, with some inner-gall cells being polytene. In plants, polytene chromosomes are normally only seen in antipodal cells, endosperm, suspensor cells and anther tapetal cells. These types of cells provide nutrients for developing cells, as do the inner-gall cells. The polytene characteristic together with the expression of a seed protein may be the first indication that gall formation triggers similar signalling pathways as in seed development.

108 Cloning and characterization of genes involved in beet cyst nematode resistance

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Cyst nematodes are major pests of crop species worldwide, e.g. potato and sugar beet. After root invasion, larvae differentiate into males and females. Females develop into cysts filled with eggs. The $Hs1^{pro-1}$ gene from *Beta procumbens* a wild relative of sugar beet prevents cyst formation thus giving full resistance to the beet cyst nematode *Heterodera schachtii*. The gene has been cloned from its position in the beet genome. It encodes a small polypeptide with 282 aminoacids with a leucine rich region and a putative membrane spanning segment. However, lack of homology to previously cloned R genes indicates that $Hs1^{pro-1}$ belongs to a new class of disease resistance genes.

An in vitro system was developed for studying the host parasite interaction which is based on the transformation with *A. rhizogenes* carrying the *rol* genes. The resulting hairy roots were grown on petri dishes and infected with *H. schachtii* juveniles. After transformation with the cDNA clone 1832, the same incompatible reaction was found as in the resistant donor line. After inoculation with two different pathotypes a race specific response was found comparable to non-transgenic beet carrying the $Hs1^{pro-1}$ gene. A 1.6 kb fragment flanking the 5' region of the gene was found to carry regulatory elements which respond to nematode attack. GUS reporter gene expression driven by this regulatory element was restricted to feeding sites of the nematode suggesting that the $Hs1^{pro-1}$-promotor is pathogen responsive. The gene has also been introduced into *A. thaliana* under the control of the 35S promotor. Twelve transgenic lines of *A. thaliana* with complete resistance to *H. schachtii* have been selected. The same resistance reaction as in sugar beet was observed and resistance was inherited as a single locus and stably transmitted to T3 progeny.